

Interaction of a C-terminal Truncated Hepatitis C Virus Core Protein with Plasmid DNA Vaccine Leads to *in vitro* Assembly of Heterogeneous Virus-like Particles

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Abstract: Recently, it has been shown that HCV core proteins (HCcAg) with C-terminal deletions assemble *in vitro* into virus-like particles (VLPs) in the presence of structured RNA molecules. Results presented in this work showed that a truncated HCcAg variant covering the first 120 aa (HCcAg.120) with a 32 aa N-terminal fusion peptide (6xHistag-XpressTM epitope) interacts with plasmid DNA vaccine. Interestingly, the buoyant density of VLPs containing HCcAg.120 in CsCl gradients changed from 1.15-1.17 g mL⁻¹ to 1.30-1.34 g mL⁻¹ after addition of plasmid DNA to assembly reactions. In addition, a delay in electrophoretic mobility of HCcAg.120-plasmid samples on agarose gels was observed indicating a direct interaction between VLPs and nucleic acids. Remarkably, addition of either plasmid DNA or tRNA to assembly reactions led to heterogeneous and larger VLPs formation than those observed in HCcAg.120 assembly reactions. VLPs containing HCcAg.120 induced a specific IgG antibodies in mice that reacted with hepatocytes from HCV-infected patients. VLPs obtained in this work would be important to elucidate the mechanisms behind the ability of HCcAg to assemble into a nucleocapsid structure. Besides, the capacity of particles containing HCcAg.120 to interact with nucleic acids could be used in the development of DNA vaccines and viral vectors based on these particles.

Key words: *E. coli*, hepatitis C, core antigen, virus like particles

INTRODUCTION

Hepatitis C virus (HCV) is a major worldwide health problem. HCV is a member of the *Hepacivirus* genus of the *Flaviviridae* family^[1]. The genome is a single-stranded positive-sense RNA of approximately 9600 nt and encodes a polyprotein with a single open reading frame (ORF) of 3008–3033 aa which is processed by host and viral proteases^[2]. The structural proteins (core, E1, E2 and potentially p7) precede the nonstructural proteins (NS2, 3, 4A, 4B, 5A and 5B) in the polyprotein. The biogenesis of HCV core protein (HCcAg) is dependent on the interaction of the signal sequence of nascent polypeptide with the endoplasmic reticulum (ER) membrane^[3]. Although it has been suggested that HCcAg function to encapsidate the viral genome within a nucleocapsid particle, little is known about the *in vivo* assembly pathway or structure of the HCV nucleocapsid and virion^[4].

Since virion nucleocapsid formation involves multimerization of HCcAg and its interaction with viral RNA, these characteristics of HCcAg have been extensively studied and specific functions mapped to

discrete portions of the molecule^[5-8]. Difficulties in obtaining mature HCcAg have required that core proteins with C-terminal deletions be used for *in vitro* functional studies^[9-11]. In addition, these HCcAg variants self-assemble into nucleocapsids in cell-free assays in the presence of structured nucleic acids^[9,11]. However, there are still many questions open about the assembly properties of HCcAg.

Previously, it has been reported that a C-terminal truncated HCcAg variant (covering aa 1-120) with a 6xHis tag fusion at N-terminus (HCcAg.120) assembled into virus-like particles (VLPs) when expressed in recombinant *Escherichia coli* cells^[10]. On the other hand, a previous study had demonstrated that a DNA vaccine containing the structural region of HCV (pIDKE2 plasmid) induces a strong humoral and cellular immune response in various animal models^[12,13]. Since particulate antigens have been proposed as potential carriers for DNA vaccines^[14], the capacity of HCcAg.120 to interact with pIDKE2 plasmid and to assemble into VLPs *in vitro* was investigated in this study.

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MATERIALS AND METHODS

Plasmid, antibodies and strain: The pIDKE2 plasmid containing the structural region of HCV (HCcAg, E1 and E2) has been previously described^[12]. It was used in protein-nucleic acid interaction studies. A mouse monoclonal antibody against the residues 5-35 of HCcAg (mAb SS-HepC.1) was used to detect HCcAg.120^[15]. Anti-HCcAg.120 IgG antibodies obtained in mice immunized with VLPs containing HCcAg.120 were used in immunoelectron and immunofluorescence microscopy analysis. The BL21(DE3)pLysS strain was used to express HCcAg.120 as previously reported^[10].

HCcAg.120 purification from recombinant *Escherichia coli* cells: HCcAg.120 was semipurified under denaturing conditions with purity higher than 85% as previously described^[10]. Later on, HCcAg.120 in denaturing buffer (DB) (8M urea, 20 Mm Tris-HCL pH 8, 250 mM NaCl) was applied to reverse-phase (RP) high-pressure liquid chromatography (HPLC) C8 column (either 4.6 x 150 mm or 10 x 250 mm) (Vydac HPLC columns, USA) as previously described^[9,16]. It was eluted using a linear gradient of acetonitrile (AcN) in 5% trifluoroacetic acid (TFA) at 1 mL min⁻¹ flow rate. The major eluted fraction containing HCcAg.120 was dialyzed overnight at 4°C against TNGU buffer (20 mM Tris [pH 7.0], 100 mM NaCl, 1% Glycerol, 2 M Urea). Later on, HCcAg.120 was concentrated by 30% ammonium sulfate precipitation and resuspended at 2 mg mL⁻¹ in TNGU buffer. Finally, HCcAg.120 was dialyzed overnight at 4°C against refolding buffer (RB) (20 mM Tris [pH 7.0], 100 mM NaCl, 1% Glycerol). Renatured HCcAg.120 sample (after a 16,000 g spin at 4°C for 15 min) was tested in three percent agarose gels prepared in 20 mM Tris-acetate-0.5 mM EDTA (TA) buffer and stained with TA buffer containing 10 µg mL⁻¹ of ethidium bromide. HCcAg.120 was purified as a single protein with purity higher than 90 %.

HCcAg.120-pIDKE2 plasmid interaction studies: *In vitro* protein-nucleic acids assembly reactions were carried out using conditions previously described^[9,11]. Ten micromolar of purified HCcAg.120 in renatured buffer was mixed with an equal volume of pIDKE2 plasmid at 100:1 protein-nucleic acid molar ratio. As control, denatured HCcAg.120 was resuspended in refolding buffer containing tRNA (Sigma) at 100:1 protein-nucleic acid molar ratio under RNase-free conditions. The reactions were incubated at 30 °C for 10 min followed by 15 min on ice and a 16,000 g spin at 4°C for 15 min.

Equilibrium density gradient centrifugation: Five hundred microlitre of either renatured HCcAg.120 or HCcAg.120-pIDKE2 plasmid samples were applied to a discontinuous 1-1.5 g mL⁻¹ cesium chloride (CsCl)

density gradient in the same buffer of the protein sample, centrifuged at 100 000 g for 26 h in a Beckmann SW40Ti rotor and fractionated. Five hundred microlitre aliquot of each fraction was collected from the bottom of the tube. Samples were also separated on a linear 25-70 % sucrose density gradient solution and centrifuged at 100 000 g for 16 h. The refractive index of each fraction was measured using an Abbe-3L refractometer (Milton Roy). To detect HCcAg.120, fractions were assayed by Dot blot as previously reported^[17]. The intensity of the resultant bands was quantified by measuring the optical density with the Eagle Eye II still video system (Stratagene).

Agarose gel electrophoresis: Samples containing either pIDKE2 plasmid or HCcAg.120-pIDKE2 plasmid were analyzed on agarose gel as previously described^[18]. The sample was mixed with 5X loading buffer (50% glycerol, 0,02% bromophenol blue) and loaded onto a 1% agarose gel with ethidium bromide (0.5 µg mL⁻¹). The gel was run in 1X TAE buffer and the gel was either detected under UV light or stained with Coomassie brilliant blue R250 (CBB, Sigma, St. Louis, USA).

Transmission electron microscopy (TEM): Either renatured HCcAg.120, or pIDKE2 plasmid, or HCcAg.120/pIDKE2 plasmid, or HCcAg.120/tRNA were fixed in glutaraldehyde and negatively stained with uranyl acetate prior to analysis by transmission electron microscopy as previously described^[15].

Detection of HCV core protein in hepatocytes: For Immunoelectron microscopy (IEM) analysis, samples of liver tissue from either HCV-infected patients or healthy individuals^[19] were fixed with 4% (v/v) paraformaldehyde containing 0.2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) at 4 °C for 3 h and washed with 0.1 M phosphate buffer. Fixed cells were dehydrated as described above, embedded in Lowicryl and polymerized by exposure to ultraviolet light at room temperature for 72 h. Ultrathin sections of liver biopsies were incubated with anti-HCcAg.120 IgG antibodies in phosphate buffer, for 45 min at RT. Specific reactions were detected as previously described^[19]. For immunofluorescence staining (IF) analysis, samples were immediately fixed with 4% paraformaldehyde in PBS at 4 °C and then mounted on gelatine-coated glass slides and stored for 2 days at -200 °C. Then samples were treated as previously described^[20] and incubated overnight at 4 °C with anti-HCcAg.120 IgG antibodies. Specific reactions were detected as described elsewhere^[20]. Stained samples were coverslipped in Vectashield mounting medium (Vector Laboratories, Inc. Burlingame, CA., USA), sealed with nail polish and viewed on an immunofluorescence microscope.

Statistical analyses: All data were presented as mean±SE. Comparison of diameters of VLPs was conducted by Kruskal-Wallis test and Dunn's post test with GraphPad PRISM 4.0 software. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

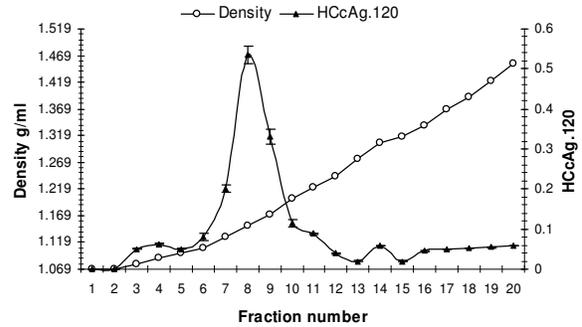
Previous studies had demonstrated that HCcAg bound to the 60S ribosomal subunit and RNA *in vitro*^[3,6,7,21]. In addition, Kunkel et al have suggested that interaction between HCcAg and nucleic acids *in vitro* plays a critical role for self-assembly of HCcAg into VLPs^[9]. They also suggested that HCcAg can undergo extensive conformational changes upon binding to nucleic acid and assembly into VLPs^[22].

On the other hand, a previous study had demonstrated that a DNA vaccine containing the structural region of HCV (pIDKE2 plasmid) induces a strong humoral and cellular immune response in various animal models^[12,13]. Since particulate antigens have been proposed as potential carriers for DNA vaccines^[14], the capacity of HCcAg.120 to interact with pIDKE2 plasmid was investigated.

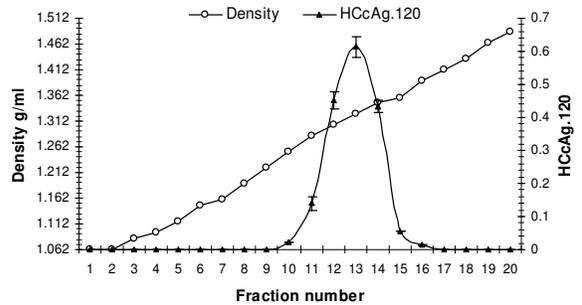
HCcAg.120 was mixed with pIDKE2 plasmid at 100:1 protein-nucleic acid molar ratio as described above. Firstly, a density gradient centrifugation was used to demonstrate a possible interaction of pIDKE2 plasmid with HCcAg.120 in the VLPs assembly reactions. We expected that VLPs containing HCcAg.120 either in the presence or in the absence of pIDKE2 plasmid should differ in buoyant density according to their nucleic acids content.

As shown in Fig. 1, the CsCl gradient was fractionated and the individual fractions assayed for the presence of HCcAg.120. A peak fraction containing the renatured HCcAg.120 in the absence of pIDKE2 plasmid that migrated to a position in the gradient corresponding to a buoyant density of 1.15-1.17 g mL⁻¹ was observed (Fig. 1A). Interestingly, the sedimentation behavior of HCcAg.120 changed from 1.15-1.17 g mL⁻¹ to 1.30-1.34 g mL⁻¹ in HCcAg.120-pIDKE2 plasmid samples, indicating a possible interaction between VLPs containing HCcAg.120 and pIDKE2 plasmid (Fig. 1B). Similarly, sucrose gradient analysis showed high density sucrose fractions containing HCcAg in HCcAg.120-pIDKE2 plasmid samples (not shown).

These high density HCcAg.120 fractions were only observed in HCcAg.120-pIDKE2 plasmid samples. This is in agreement with previous results showing that native core-like particles containing nucleic acids obtained from recombinant *E. coli* and *Pichia pastoris* cells migrate with buoyant densities values ranging from 1.28 g mL⁻¹ to 1.32 g mL⁻¹ in CsCl and sucrose gradients^[10,11,15]. It is interesting to note that native nucleocapsid particles from sera of HCV-infected patients show high buoyant density values in sucrose



A



B

Fig. 1: CsCl gradient centrifugation analysis. The CsCl density of each fraction is shown (open circles; density (left ordinate) expressed in grams per milliliter). Detection of HCcAg.120 was analyzed by densitometry of dot blot (closed triangles; HCcAg.120 (right ordinate) expressed as signal (OD) at 620 nm). A) Analysis of renatured HCcAg.120. B) Analysis of HCcAg.120-pIDKE2 plasmid samples

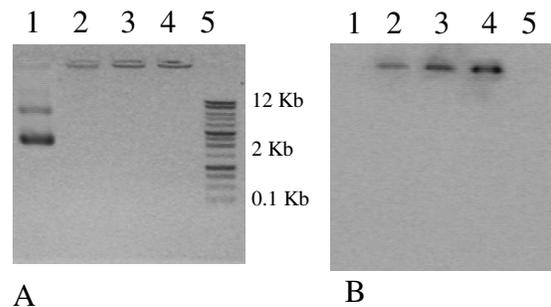


Fig. 2: Presence and characteristics of HCcAg.120-pIDKE2 from CsCl fractions (12-14). 1% agarose gel of the HCcAg.120-pIDKE2 complexes visualized with ethidium bromide (A) and Comassie Blue (B). The lanes contain: pIDKE2 plasmid (1); CsCl fractions (12-14) containing HCcAg.120-pIDKE2 complexes (2-4, respectively); 1 Kb PlusLadder™ (Gibco BRL) size markers (5)

and CsCl gradients^[4,23]. In addition, nucleocapsid particles obtained after detergent treatment from sera of chimpanzees and humans with chronic non-A, non-B hepatitis, have shown similar buoyant density values^[4,24]. Data suggest that samples containing HCcAg.120-pIDKE2 plasmid showed similar buoyant density values to those previously observed for nucleocapsids found in HCV-infected patients.

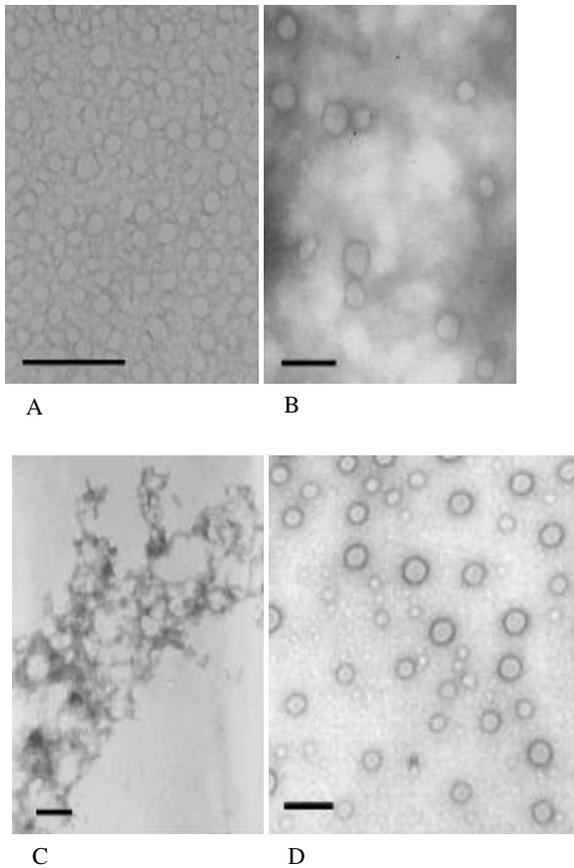
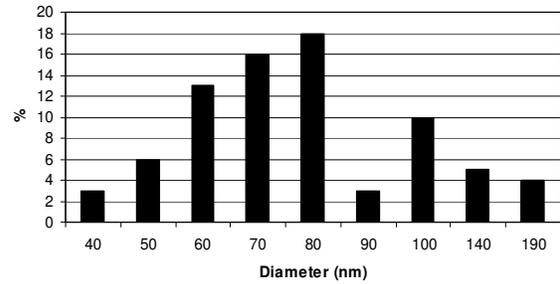


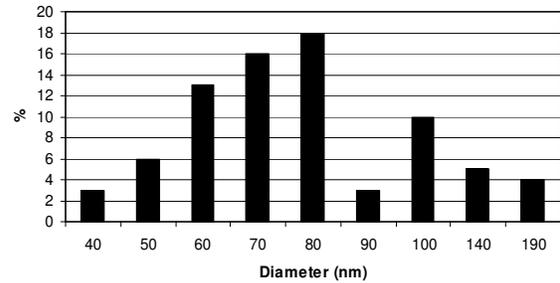
Fig. 3: Transmission electron microscopy images of samples containing HCcAg.120. Micrograph of negatively stained samples containing: A) CsCl fractions (1.15-1.17 g mL⁻¹) containing renatured HCcAg.120 B) HCcAg.120-pIDKE2 plasmid C) pIDKE2 plasmid D) HCcAg.120-tRNA. Bar 200 nm

Afterwards, gradient fractions of HCcAg.120-pIDKE2 plasmid samples were electrophoresed on 0.5% agarose gels and stained with EtBr. The formation of HCcAg.120-pIDKE2 plasmid complexes are shown in Fig. 2. Intense EtBr staining was seen in all the cases. A delay in electrophoretic mobility of HCcAg.120-pIDKE2 samples was observed (Fig. 2A). EtBr-stained bands were also positive for Coomassie staining, indicating that these were indeed HCcAg.120-pIDKE2 complexes (Fig. 2B).

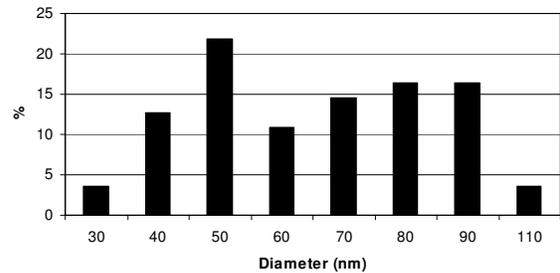
In addition, assembly reactions containing HCcAg.120 were analyzed by TEM. Homogeneous particles with an average diameter of approximately 30 nm (29.91 nm, SD 5,84) were observed for gradient fractions containing renatured HCcAg.120 (1.15-1.17 g mL⁻¹), indicating that CsCl and sucrose gradient conditions did not affect the VLPs (Fig. 3A). Figure 4A shows the frequency distribution of particle diameters. The diameters of these particles ranged from 20 to 41 nm. Remarkably, renatured HCcAg.120 (present in HCcAg.120-pIDKE2 plasmid samples) assembled into larger and heterogeneous VLPs (average diameter of 78.63 nm, SD 29.59) (Fig. 3B and 4D).



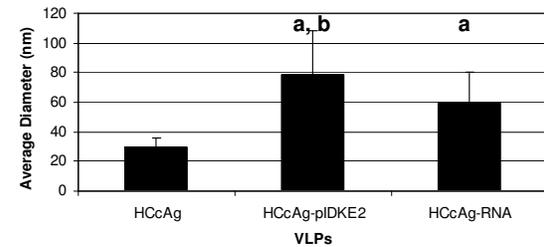
A



B



C



D

Fig. 4: Histogram representing the frequency distribution of particle diameters observed by TEM in the assembly experiments of HCcAg.120. The diameter of particles was precisely measured in at least 10 fields (from 250 to 500 VLPs) from electron micrographs. Diameters of VLPs derived from renatured HCcAg.120 (A) or HCcAg.120-pIDKE2 plasmid (B) or HCcAg.120-tRNA (C) are shown. (D) Comparison of diameters of VLPs from different *in vitro* assembly reactions containing HCcAg.120. (a) HCcAg-pIDKE2,HCcAg-tRNA>HCcAg, p<0.001; (b) HCcAg-pIDKE2>HCcAg-tRNA, p<0.05

Figure 4B shows the frequency distribution of particle diameters. The diameters of these particles ranged from 36 to 190 nm. However, these VLPs were not observed

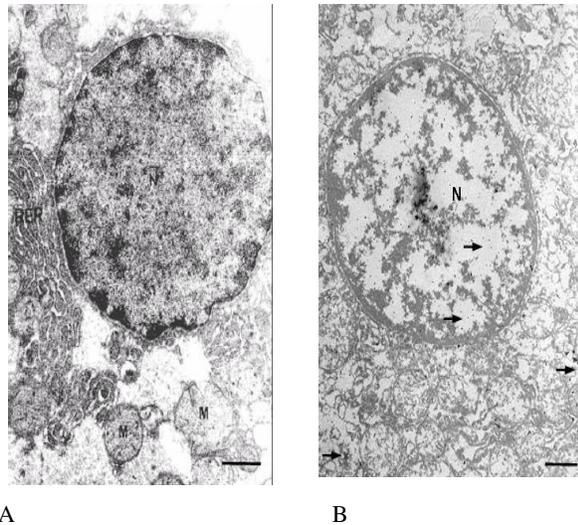


Fig. 5: Immunolabeling of HcAg in hepatocytes of liver biopsies from HCV-infected patients by IEM. A) No immunostaining was observed in hepatocytes of liver biopsies from healthy individuals. B) Immunostaining (arrows) of liver sections from HCV-infected patients revealed HcAg in cytoplasm and nucleus of hepatocytes. Nucleus (N); Mitochondria (M); Rough endoplasmic reticulum (RER). (Bar=1 μ m)

in pIDKE2 plasmid samples (Fig. 3C). It is interesting to note that HcAg.120 also assembled into heterogeneous VLPs in the presence of tRNA molecules (average diameter of 59.89 nm, SD 20.16) (Fig. 3D and 4C). The diameters of these particles ranged from 25 to 100 nm. These particles were larger than those obtained in HcAg.120-only assembly reactions but shorter than those observed in HcAg.120-pIDKE2 plasmid assembly reactions (Fig. 4D).

Finally, IEM (Fig. 5) and IF analysis (not shown) showed that anti-HcAg.120 IgG antibodies, obtained in mice immunized with VLPs containing HcAg.120 (not shown), specifically reacted with hepatocytes from HCV-infected patients but not with hepatocytes from healthy individuals. This suggests that these VLPs share epitopes with the HcAg expressed during HCV infection in hepatocytes.

Previously, *in vitro* assembly of a C-terminal truncated HcAg (covering the first 124 aa) into VLPs using a purified recombinant protein from *E. coli* was observed only in the presence of structured RNA^[9]. These VLPs were heterogeneous in size and larger than the expected native HCV nucleocapsids. HcAg.120 assembled into homogeneous VLPs in the absence of structured nucleic acids. However, addition of either pIDKE2 plasmid or tRNA molecules to assembly reactions led to larger and heterogeneous VLPs which appeared similar to those previously described^[9]. One hypothesis is that the denaturation-renaturation

process partially affected the folding of the protein which leads to the formation of larger agglomerates in the presence of nucleic acids. Indeed a recent report describes the purification of various variants of HcAg under native conditions^[11]. HcAg assembled *in vitro* into VLPs with similar size and appearance to native HCV nucleocapsids in the presence of structured RNA. In addition, only the first 79 N-terminal aa of HcAg assembled *in vitro* into homogeneous particles of regular size and shape in the presence of structured tRNA. However, a few contaminants copurified with the recombinant proteins and their contribution to the VLPs assembly should be studied^[11].

Another hypothesis would be that nucleic acids misdirect *in vitro* assembly of VLPs. Misdirectors are postulated to disturb the geometry of intersubunit contacts and convert a growing spherical polymer, which will incorporate only a few hundred subunits, into a polymer that can incorporate an unlimited number^[25,26]. A misdirector not only should prevent subunits from forming spherical particles but also could lead to the formation of regular nonnative structures^[25,26]. Thus, it is possible that misdirection of VLPs assembly by nucleic acids leads to large and heterogeneous VLPs formation.

Therefore, it is not clear why HcAg assembled *in vitro* in the presence of nucleic acids into particles larger than those isolated from infected subjects. *In vivo*, it has been suggested that HcAg interaction with cell membranes or lipids and/or envelope glycoproteins may be required for correct viral particle morphogenesis^[18,27]. So, it is possible that some cellular factors might be required for proper *in vitro* assembly of nucleocapsid-like particles.

CONCLUSION

Results presented in this work showed that interaction of HcAg.120 with plasmid DNA and tRNA leads to *in vitro* assembly of heterogeneous VLPs. This approach would be important to elucidate the mechanisms and cellular factors that might be required for proper *in vitro* assembly of nucleocapsid-like particles. Besides, the capacity of particles containing HcAg.120 to interact with nucleic acids could be used in the development of DNA vaccines and viral vectors based on these particles.

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