



Characterization of influenza H1N1 Gag virus-like particles and extracellular vesicles co-produced in HEK-293SF



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ABSTRACT

One of the concerns associated with the use of influenza virus-like particles (VLPs) as vaccine candidate or delivery system is their heterogeneous composition. Enveloped VLPs take up the host cell membrane at the budding site carrying out not only the viral antigenic proteins but also host proteins. In addition, the intrinsic nature of cells to produce membrane derived vesicles or extracellular vesicles (EVs), which have similar size to the VLPs, makes VLP purification process challenging. To further characterize these particles and identify proteins that are unique to each population, comparative proteomic analyses were completed to ultimately provide guidance for rational design of separation protocols. The VLPs were produced in suspension and serum free media by transient transfection of an inducible clone of a Human Embryonic Kidney (HEK-293SF) cells expressing HA and NA (H1N1/A/Puerto Rico/8/34), with a plasmid containing the gag gene of HIV-1 fused to GFP. EVs were produced independently from the non-transformed HEK-293SF cell line as a control for comparative studies. Both preparations were characterized for total nucleic acids and protein concentrations and extensively analyzed by nanoLC-MS/MS for their protein compositions. The proteomic analyses showed that aside from the recombinant VLP proteins, nucleolin was the most abundant host cell protein uniquely identified within VLPs (considering the MASCOT score value) while lactotransferrin and heat shock protein 90 were the most abundant proteins in EVs. Overall, this comparative study identifies potential target proteins as specific markers to guide VLP purification and discusses the biogenesis of enveloped particles released in HEK-293 cell suspension cultures emphasizing on the biological functions of host cell proteins identified.

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1. Introduction

Virus-like particles (VLPs) constitute an attractive platform to present a repetitive array of dominant antigens while preserving the protein native conformation. VLPs lack viral genome and their structure mimics wild-type viruses, therefore they are safer and more immunogenic than virus-based and subunit vaccines, respectively [1]. Similarly to viruses, enveloped VLPs bud from the cell membrane taking up the host cell membrane at the budding site [2]. In addition, the formation of VLPs relies on host cell protein machinery for gene transcription, translation, protein translocation throughout the cytoplasm, assembly and budding. Hence, released VLPs do not only contain the recombinant viral proteins of interest,

but also several host cell proteins as well as genomic RNA and DNA [3]. The expression system employed for VLP production will determine the nature of the host cell derivatives within the VLPs.

The production of influenza VLPs has been explored in several expression systems including insect cells [4], plants [5], and mammalian cells [6]. Within mammalian cells, the human embryonic kidney cell line HEK-293 seems attractive for VLP production since, in addition of offering human-like post-translational modifications, they are easy to transfect and show efficient growth in suspension culture in serum-free medium [7]. However, there remain some concerns about the use of HEK-293 cells for vaccine manufacturing and/or viral vector production for gene therapy. Being a human derived cell line introduces a risk of contamination with human-specific viruses and a tumorigenic potential has been attributed to this cell line [8,9]. Nonetheless, several biopharmaceuticals

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produced in HEK293 cells have been approved by the FDA for therapeutic use [10].

Another aspect to consider when producing influenza VLPs in mammalian/human cells is the co-production of extracellular vesicles (EVs) which are very similar in size to VLPs. The secreted vesicles are diverse in nature and can be differentiated in three types: exosomes, shedding microvesicles and apoptotic bodies [11]. The exosomes have an intracellular origin and a size in the range of 30–150 nm. Shedding microvesicles bud from the cell membrane with a size ranging from 50 to 1000 nm and the apoptotic bodies (50–5000 nm) have more irregular shapes and arise from apoptotic cells outward membrane blebblings [12]. Exosomes play an important role in cell-cell communication and have received a lot of attention in the last decades for different applications as cancer biomarkers, as well as drug delivery vehicles and chemotherapeutic sensitization agents [13,14]. Exosomes contain intra-lumen proteins and genomic material that snapshot the metabolic state of the secreting cell [15]. The formation of exosomes starts with early endosomes that subsequently form multi-vesicular bodies (MVBs) containing intraluminal vesicles (ILVs). Then, the MVBs fused with the plasma membrane and the ILVs or exosomes are released to the extracellular milieu. The endosomal sorting complexes required for transport (ESCRT) mediate the formation and scission of MVBs [11]. Enveloped viruses, like (Human immunodeficiency virus) HIV-1, “hijack” this cell mechanism for its viral budding [16].

In light of these facts and considering that enveloped VLPs are membrane-enclosed multimeric protein complexes, even after an intensive purification and polishing, host cell proteins will not be completely removed since they form part of the particles. In addition, the presence of EVs in the final preparations, due to similarities in size and structure with VLPs, should be considered. An extensive characterization of these particles will provide more insights on the nature of their unique components which could be used for selection of purification strategies or process intensification and will contribute to further elucidate the intracellular mechanisms (transport, assembly, and budding) involved in the biogenesis of these enveloped particles. In this study, we analyzed the nucleic acid and total protein content and provided an extensive comparative proteomic analysis assessing the protein composition of influenza H1N1 Gag-VLPs and extracellular vesicles co-produced in HEK-293SF.

2. Materials and methods

2.1. Production of VLPs and EVs

The VLPs analyzed in this study were produced in a HEK-293 inducible stable cell line expressing hemagglutinin and neuraminidase of A/Puerto Rico/8/1934 (H1N1) subtype (293HA-NA). The VLPs formation was mediated by transient transfection of a plasmid containing Gag protein of HIV-1 fused to GFP as reported previously [17]. The supernatant of cells, 72 h post induction, was concentrated and semi-purified by ultracentrifugation through 25% sucrose cushion. Subsequently, the recovered particles were ultrafiltrated by Tangential flow filtration (TFF) through a membrane of 1000 KDa cut-off to remove smaller extracellular vesicles and cell debris. This sample is called throughout this work as “VLPs-EVs” referring to the fact that this sample might yet contain EVs that were co-produced with the VLPs.

The extracellular vesicles were produced from the cell line HEK-293CymRrcTA cells [17]. The supernatant was harvested at 72 h post-induction with cumate and 25X concentrated by ultracentrifugation through a 25% sucrose cushion. In the present work this sample is called “only EVs”.

2.2. nLC-MS/MS of tryptic digests

“VLPs-EVs” and “only EVs” samples were reduced with 4 mM dithiothreitol for 1 h at 56 °C, and then alkylated with 20 mM iodoacetamide for 1 h at room temperature in the dark. The samples were subsequently digested with trypsin at a 30:1 protein to enzyme ratio overnight at 37 °C. Peptides samples were then analyzed by reversed phase nano liquid chromatography tandem mass spectrometry (nLC-MS/MS), using a nanoAcquity UPLC (Waters) coupled to an Orbitrap XL mass spectrometer (Thermo Fisher) using electrospray ionization (ESI). First, the peptides were loaded onto a 50 mm × 300 μm C8 (Dionex) and a 20 mm × 180 μm C18 (Waters) trap columns in series, and then eluted onto a 100 mm × 100 μm C18 analytical column (Waters). The elution gradient was linear: 1–45% solvent B over 18 min, 45–85% solvent B over 3 min, 85–1% solvent B over 1 min. The columns were finally re-equilibrated at 1% solvent B for 8 min. Solvent A was 0.1% formic acid in HPLC grade water; Solvent B was 0.1% formic acid in acetonitrile. Data-dependent MS/MS analysis was performed, and the peak list files were searched against a database containing the Uniprot Human and influenza strain H1N1 A/PR sequences using MASCOT search engine (version 2.5; Matrix Science). The significance threshold was set to $p < 0.05$. Three technical replicates were performed. The selection criteria for the data shown in this study was set as follow: Unique peptide matches were accepted when MASCOT score was greater than 50 and sequence coverage >4%.

2.3. Total protein, RNA, DNA and virus particle quantification.

The nucleic acid extraction from “VLPs-EVs” sucrose cushion concentrated samples was performed using the high pure viral nucleic acid kit (Roche, Switzerland). For RNA quantification, a DNase I (ThermoFisher, USA) treatment was performed to remove interfering DNA (14U/200 μl). For DNA quantification, samples were treated, before extraction, with benzonase nuclease (E1014-25KU, SigmaAldrich, USA) 125 U/ml in order to only measure the DNA content inside “VLPs-EVs” and “only EVs”. Finally, the PicoGreen Quant-it (ThermoFisher, USA) and Ribogreen (Molecular Probes, USA) assay were used to measure the DNA and RNA concentration, respectively. Three replicates were included for each nucleic acid extraction. The total protein concentration was estimated by DC™ Protein Assay (Bio-Rad, USA).

The quantification of VLPs is reported as Gag-GFP fluorescent events/ml and was estimated using a BD LSR-Fortessa SORP flow cytometry system (Becton-Dickinson, USA) as previously published [18].

2.4. Transmission electron microscopy

The Transmission electron microscopy was performed using a Hitachi H-7500 TEM operated in high contrast mode at an acceleration voltage of 80 kV following the methodology described in [17].

3. Results and discussion

The VLPs characterized in this study were produced in the 293HA-NA stable cell line after transient transfection of a plasmid containing the Gag gene of HIV-1 fused to GFP. The supernatant was 25X concentrated through a 25% sucrose cushion and diafiltered by TFF. This preparation throughout the manuscript is referred to as “VLPs-EVs”. On the other hand, the extracellular vesicles (EVs) were produced from the cell line HEK-293CymRrcTA cells after induction with cumate and were 25X concentrated

through a 25% sucrose cushion. In this work this preparation is referred to as “only EVs”.

3.1. Total protein, nucleic acid quantification and TEM

The quantification of total protein, RNA and DNA within “VLPs-EVs” and “only EVs” samples is shown in Table 1. The nucleic acid concentration corresponds to the inside content of “VLPs-EVs” and “only EVs” since the samples were pre-treated with benzonase before RNA and DNA extraction. The result of the DNA quantification shed light on the need for a better follow up of the DNA content present in cell-derived influenza VLPs during the downstream processing until final formulation. The DNA contained within the particles will be difficult to remove even after implementing sophisticated purification steps and the WHO recommends 10 ng DNA/vaccine dose (15 µg of HA one dose) [19]. However, downstream process optimization can help to reduce DNA released in the supernatant and attached to the particles. Multiple benzonase treatments combined with size exclusion chromatography might contribute to efficiently eliminate free and external DNA that remain attached to the particles; keeping in mind that the use of benzonase increases process cost. The concentration of hemagglutinin (HA) in our “VLPs-EVs” sample was determined by Single Radial Immunodiffusion (SRID) as 9.2 µg/mL [17]. Also, the VLPs were quantified by Flow Cytometry (Table 1) such that the nucleic

acid content can be related to the number of particles. Furthermore, the two preparations “VLPs-EVs” and “only EVs” were observed by Transmission Electron Microscopy (TEM) (Fig. 1). The micrographs of “VLPs-EVs” showed particles with more heterogeneity in shape and size at around 80–250 nm. On the other hand, for sample “only EVs”, the shape and size of particles were more consistently homogeneous. All EVs identified were around 100 nm.

3.2. nLC-MS/MS

In the present study we have analyzed samples by nLC-MS/MS to reveal the protein composition of these particles. The proteins uniquely identified in the “VLPs-EVs” and “only EVs” samples are presented and discussed in separated sections and tables. All proteins detected in the “VLPs-EVs” sample are shown in Table 2 as unique and common proteins compared with “only EVs” sample. The proteins were grouped depending on their biological function. Mainly, proteins related to cell cytoskeleton, transport, RNA and DNA binding and processing, and post-translational modifications were identified in both samples.

3.2.1. Proteins identified in VLPs-EVs

– Heterologous proteins

As unique proteins present in the “VLPs-EVs” sample, the four recombinant proteins that were inserted to mediate VLP formation were identified (HA, NA, Gag-GFP).

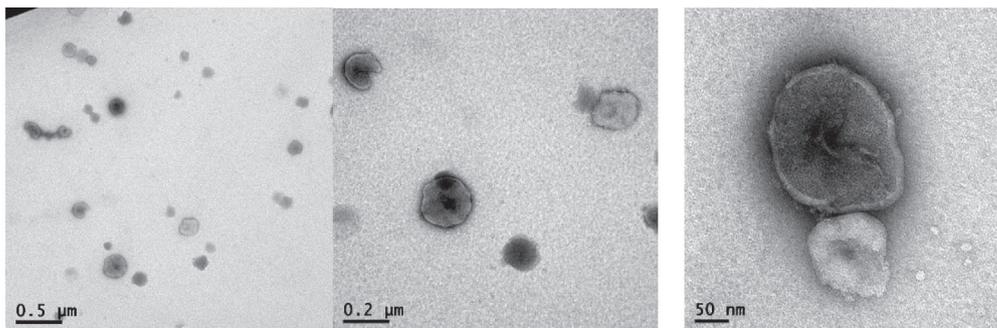
– RNA binding and processing

The nucleolin protein, one of the most abundant proteins in the nucleolus, was uniquely identified in “VLPs-EVs” sample with a MASCOT score of 324 (Table 2). This RNA-binding protein inter-

Table 1
Quantification of total protein, DNA and RNA contaminants.

	VLPs-EVs	Only EVs
Total protein (µg/ml)	400 ± 6	310 ± 30
DNA (µg/ml)	1.9 ± 0.2	0.6 ± 0.03
RNA (µg/ml)	36.5 ± 3.7	44.7 ± 2.4
Gag-GFP events/ml	7.22 × 10 ⁹	N/A

VLPs-EVs



“Only EVs”

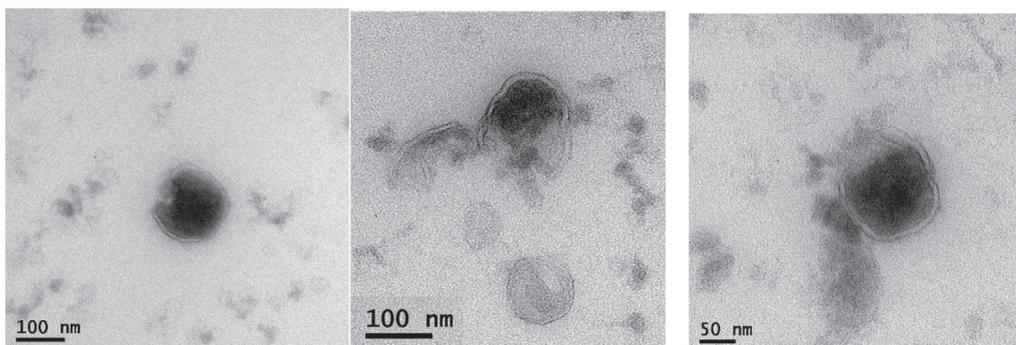


Fig. 1. Transmission electron microscopy (TEM) images of “VLP-EVs” and “only EVs” preparations.

Table 2

Proteins identified uniquely in VLPs-EV sample and common proteins identified in VLPs-EVs and “only EVs” samples.

	Proteins only identified in “VLPs-EVs”			Common proteins identified in “VLPs-EVs” and “only EVs”				
		MASCOT Score	Predicted MW (Da)	Peptide no.	MASCOT Score	Predicted MW (Da)	Peptide no. <small><i>n</i>/VLPs-EVs/ only EVs*</small>	
Heterologous proteins	Pr55(Gag) [Human immunodeficiency virus 1]	3535	55,894	24				
	Green fluorescent protein	229	26,869	6				
	Hemagglutinin [Influenza A virus (A/Puerto Rico/8/1934 (H1N1))]	148	63,341	8				
	Neuraminidase [Influenza A virus (A/Puerto Rico/8/1934 (H1N1))]	61	50,111	2				
Cytoskeleton associated proteins					Tubulin β -chain	641/1015	49,639	13/18
					Actin, cytoplasmic 1	724/752	41,710	13/13
RNA binding and processing	Nucleolin	324	76,568	8	Heterogeneous nuclear ribonucleoprotein A1	62/95	19,460	1/1
	Heterogeneous nuclear ribonucleoproteins C1/C2	161	31,929	3				
Histones and Histone regulation proteins	Protein SET	51	31,105	1	Histone H4	767/599	11,360	7/9
					Histone H2A	493/484	18,470	5/6
					Histone H2B	386/637	13,898	5/6
					Histone H1.3	176/386	22,336	3/6
					Histone H3	113/144	14,905	4/5
					Histone H3.1	124/118	15,404	2/2
					Histone H3.2	217/238	15,379	5/5
Heat shock proteins					Heat shock 70 kDa protein 1B	773/1292	70,066	15/22
					Heat shock cognate 71 kDa protein	221/237	20,000	3/5
Post-translational modifications	Peptidyl-prolyl cis-trans isomerase	322	18,001	7	Elongation factor 1- α 1	136/189	47,853	3/5
					Eukaryotic initiation factor 4A-1	122/96	20,675	1/1
					T-complex protein 1 subunit β	104/174	56,771	1/2
					GTP-binding nuclear protein Ran	94/78	26,799	3/3
Proteins involved in lipid metabolism or clearance					Clusterin	157/222	52,461	4/6
					Fatty acid synthase	76/102	273,026	2/6
Tetraspanin					CD81	71/158	24,423	1/2
Glycolytic enzymes					Glyceraldehyde-3-phosphate dehydrogenase	341/511	36,030	8/11
					Pyruvate kinase	155/508	53,011	2/8
					α -enolase	201/648	47,139	3/8
Other proteins					Lactotransferrin	83/4003	76,577	50/3
					Creatine kinase B-type	99/256	24,101	1/6

* MASCOT score and peptide no. is given as “VLPs-EVs”/“only EVs”.

acts with the nucleocapsid (NC) of Gag protein which is essential for the assembly [20] and budding of HIV-1 particles [21]. Nucleolin is also expressed on the cell surface [22]; cell-surface nucleolin and lipid rafts were shown to be involved in the virus entry process of HIV [23]. The heterogeneous ribonucleoproteins (hnRNPs) C1 and C2 were also incorporated in the influenza “VLPs-EVs”, but not in the “EVs only”. The hnRNPs bind to pre-messenger RNA protecting the RNA from being degraded by endo- or exo-nucleases and transporting it to the ribosomes for translation. This protein complex comprises a large family of over 20 members, some hnRNPs like A1, which was identified in both samples “VLPs-EVs” and “only EVs”, stay attached during RNA export before shuttling back into the nucleus [24]. The interaction between nucleolin and hnRNP C with the 3'-UTR of the amyloid mRNA precursor has been reported [25]. It is likely that Gag protein associates with these strong RNA binding proteins to

guarantee the export of the vRNA into virions and reduce the release of empty virions during HIV infection.

– Cytoskeleton associated proteins

Our analysis also revealed the presence of several proteins related to the cytoskeleton, most of these proteins were identified within both samples “VLPs-EVs” and “only EVs”, like Tubulin β -chain and Actin cytoplasmic 1. In general, the cytoskeletal proteins have been implicated in the transport of viruses throughout the cell cytoplasm to the assembly and budding site. Gag protein directly associates with actin microfilaments; in fact, the HIV-1 virions release depends on an intact actin-cytoskeleton [26,27]. In the present study, similar cytoskeletal proteins were identified within both samples, which is expected considering the co-presence of EVs in final VLP preparations. In addition, both

particles bud from the cell membrane relying on key cytoskeletal proteins for the translocation process for budding.

– Histones and Histone regulation proteins

Several types of histones were identified within “VLPs-EVs” and “only EVs” (Histone H4, H2A, H2B, H1.3, H3, H3.1, H3.2) while histone regulator protein (Protein SET) was only found in “VLPs-EVs”. Histones are highly alkaline proteins rich in lysine and arginine that form a core around which DNA is wrapped, forming the nucleosome. In humans, this histone core is organized in octamers with a central tetramer of histones H3 and H4, flanked by two dimers of H2A and H2B. The histone H1 acts as a linker between two nucleosomes [28]. Histones play an essential role in regulating transcription and the condensation of chromatin. The presence of nucleosome fragments (histones and DNA) in the extracellular environment has been associated with cell death, cellular damage and apoptosis. However, it has been reported that different mechanisms are involved in the release of exosomes and apoptotic bodies and that both vesicle species are biochemically and morphologically different. The presence of histones in exosomes might be due to the co-production of apoptotic bodies in the cell culture [29]. The Su(var)3–9, Enhancer-of-zeste, Trithorax (SET)-domain protein was only identified in “VLPs-EVs”. This protein mediates the methylation of histone lysines regulating gene expression. Depending on the specific position in the chromatin of the methylated lysine, the genes will be repressed or activated [30,31].

– Heat shock proteins

The heat shock proteins (HSPs) (Heat shock 70 kDa protein 1B, Heat shock cognate 71 kDa protein) were identified in both “VLPs-EVs” and “only EVs”. This family of proteins have been found to be released under cellular stress and they are highly conserved among different organisms. The HSPs act as chaperones, participating in protein synthesis, folding, translocation and preventing protein aggregation. They also mediate ubiquitination of aberrant proteins cooperating with the proteasome system during quality control [32]. The presence of heat shock proteins has been previously identified in exosomes, HIV-virions, influenza VLPs and influenza virus produced in Vero cells and Moloney murine leukemia virus [6,11,24,33,34].

– Post-translational modifications

Several proteins implicated in post-translational modifications were recognized in the “VLPs-EVs” and “only EVs” (elongation factor 1- α 1 (EF1 α), Eukaryotic initiation factor 4A-I, T-complex protein 1 subunit β), whereas the Peptidyl-prolyl cis-trans isomerase A was uniquely identified in the “VLPs-EVs”. The EF1 α is a crucial part of the cellular translation machinery. It is responsible for the GTP-dependent binding of aminoacyl-transfer RNA (aa-tRNA) to ribosomes, guaranteeing fidelity and rate of elongation during translation. The EF1 α interacts with actin filaments of the cytoskeleton and is also involved in cell proliferation and compartmentalization of translation in the cytoplasm [35]. This elongation factor specifically interacts with Gag protein of HIV-1, while it is not incorporated in non-lentivirus virions like Moloney murine leukemia virus [36]. However, in our study, this protein was detected in both “VLPs-EVs” and “only EVs”, which suggests that the interaction with the actin cytoskeleton results in incorporation of EF1 α into both nanoparticles species. In the case of “VLPs-EVs”, EF1 α most probably also interacts with Gag.

The eukaryotic initiation factor 4A-I (eIF4-A) is required for the binding of ribosome subunits 40S to the mRNA during the

initiation of protein biosynthesis. It has a single-stranded RNA-dependent ATPase activity, which mediates the unwinding of the 5'-proximal mRNA secondary structure facilitating the attachment to the ribosomes [37]. The T-complex protein 1 is a hetero-oligomeric protein containing of at least eight subunits that assists the correct folding and functional conformation of proteins in the cytosol of eukaryotic cells. It is involved in the folding of actin and tubulin [38].

The Peptidyl-prolyl cis-trans isomerase A, also known as cyclophilin A, catalyzes the cis-trans isomerization of peptidyl-prolyl bonds in oligopeptides, accelerating the folding of proteins [39,40]. The HIV-1 Gag protein specifically binds to cyclophilin A and B and this interaction appears to play an essential role in HIV-1 infection and replication cycle [41]. The presence of cyclophilin A only in “VLPs-EVs” (Table 2) is likely due to the interaction with Gag, which enhances the presence of the protein within the VLPs. Nevertheless, cyclophilin A has also been detected in influenza virus produced in Vero cells [33].

– Proteins transport

The GTP-binding nuclear protein Ran or Ras-related nuclear protein was identified within the “VLPs-EVs” and “only EVs”. This protein participates in the import and export of RNA and proteins between the nucleus and the cytoplasm and also its interaction with other proteins seems to play a role in the mitotic cycle [42].

– Metabolism and clearance of lipids

Proteins involved in lipid metabolism and clearance were identified either in “VLPs-EVs” and “only EVs” (Clusterin and fatty acid synthetase). Clusterin protein is a heterodimeric disulfide-linked glycoprotein that is implicated in several biological events, such as lipid transport, apoptosis, membrane lipid recycling and also it acts as a chaperone for correct folding and aggregation of secreted proteins [43]. On the other hand, the fatty acid synthetase has been previously detected in influenza virions [33] and is a multifunctional enzyme that catalyzes the formation of long-chain fatty acids from acetyl-CoA, malonyl-CoA and NADPH. It has been reported that the infection with human cytomegalovirus (HCMV) upregulates the host cell metabolism (glycolysis, TCA and fatty acid biosynthesis) to provide energy for virus replication and the inhibition of fatty acid biosynthesis during HCMV and influenza A infection leads to the suppression of viral replication [44].

Tetraspanins

The tetraspanin member CD81 was found to be associated with the “VLPs-EV” and “only EVs”. Tetraspanins constitute a superfamily of proteins that have four transmembrane domains and two extracellular loops that are organized in the membrane as enriched-tetraspanin microdomains (TEMs). While tetraspanins are considered a biomarker for exosomes [45], membrane zones enriched in tetraspanins are the areas from which HIV-1 assembles and buds [46]. The tetraspanin CD81 is well known as a co-receptor for the entry of hepatitis C virus [47] and it has also been identified in several virus such as Moloney murine leukemia virus [34], feline immunodeficiency virus, canine distemper virus, influenza virus and influenza VLPs produced in Vero cells [6,33]. These findings suggest that envelope viruses, VLPs and exosomes could follow similar path for budding through the TEMs spots.

– Glycolytic enzymes

Three enzymes involved in ATP generating steps of the glycolysis were identified in the “VLPs-EVs” and “only EVs”

(GAPDH, pyruvate kinase and α -enolase). The presence of these glycolytic enzymes in viruses, VLPs and exosomes has been previously reported [6,24,33,48]. It is still unclear why these proteins remain in the VLPs and exosomes, however alternative functions related to the regulation of virus RNA transcription have been reported for these proteins [33]. In addition, it has been shown that the administration of mesenchymal derived-stem cells exosomes after myocardial ischemia/reperfusion injury increased ATP levels, decreased oxidative stress and activated cardioprotective pathways, thereby significantly reducing infarct size. The increase of ATP levels in the injured zone was claimed to be mediated by the glycolytic enzymes which reverted the depletion of ATP characteristic of this type of injury [48]. Perhaps the presence of glycolytic enzymes in “only EVs” and “EVs-VLPs” has to do with the life span and/or bioactivity of these nanoparticles.

– Other proteins

Other proteins with diverse functions were identified in both “VLPs-EVs” and “only EVs”. Lactotransferrin (LTF) is a protein that has antiviral and antibacterial activity. Interestingly, LTF was the most abundant protein in the “only EVs” evidenced by a highest MASCOT score among the proteins identified in extracellular vesicles. LTF has been previously identified in vesicles derived from urinary exosomes of both prostate cancer patients and controls [49]. It is worth to note that the use of LTF as an additive in the HyClone SFM4Transfx-293 medium that was employed in this work to grow the HEK-293SF cells was demonstrated by MS/MS after cutting a high intensity 80 kDa band from an SDS-PAGE gel with only medium (data not shown). However, considering that the extracellular vesicles were isolated by ultracentrifugation through a 25% sucrose cushion, there should not be free LTF protein remaining in the final pellet, unless the protein forms aggregates and/or gets attached to EVs.

3.2.2. Proteins uniquely identified in extracellular vesicles

The HSP90 was uniquely identified in “only EVs” and was the most abundant, within the unique proteins, based on the MASCOT score value (Table 3). The HSP90 protein binds to a wide variety of key proteins being implicated in several biological processes and has been identified in exosomes derived from different tumorigenic cell tissues [50,51]. It has been reported that the chaperone HSP70 is the one that is specifically incorporated in HIV-1 virions

through interaction with Gag protein [34,52]. Influenza viruses produced in Vero cells did not incorporate either HSP70 and/or HSP90 [33] while influenza VLPs produced in the same cell line incorporated both [6]. In this study HSP90 was not incorporated in the VLPs or either the EVs co-produced with the VLPs.

Within the proteins identified that are involved in signalling transduction, members of 14-3-3 proteins (epsilon and theta) were only detected in “only EVs”. The 14-3-3 proteins are a family of highly conserved acidic proteins comprising several isoforms (α - η) [53]. They play a role as modulators of Ras signalling pathway [54]. The Ras protein family are small GTPases involved in cellular signals transmission in the cytoplasm. Different members of the family have been identified in extracellular vesicles released from several cell types and it is considered an EV membrane marker in the Exocarta database.

In addition, proteins associated to the ESCRT complex were uniquely detected in “only EVs” produced in HEK-293SF, such as Ubiquitin-like modifier-activating enzyme 1. The ESCRT complex is formed by four components: ESCRT-0, I, II, and III. The ESCRT-0 complex is recruited to the early endosome which at the same time recruits ESCRT-I and ESCRT-II. The complexes ESCRT 0, I and II bind ubiquitinated membrane proteins to be degraded in lysosomes, recycled or released to the extracellular milieu. Then, the endosomal membrane undergoes an inward budding to generate ILVs which requires the ESCRT-III complex for the final membrane bend and scission.

The lactadherin (MFGE8) is a protein abundantly released in mammalian glands but it has also been identified in other types of tissues, such as lung, brain and kidney. It binds to phosphatidylserine and has been detected on the surface of apoptotic bodies and exosomes produced from murine dendritic cell line (D1) and monkey kidney cell line, COS-7 [55]. The MFGE8 plays a role in the secretion of lipids.

4. Conclusions

In a previous study we have shown the feasibility of producing influenza VLPs in HEK-293SF cells and the VLP efficacy as vaccine in mice [17]. In the present work, we have characterized the composition of those influenza Gag-VLPs generated using an inducible HEK-293 producing cell line [17] and extracellular vesicles (EVs) produced from non-transformed HEK-293SF. The characterization

Table 3
Proteins uniquely identified in extracellular vesicles produced in HEK-293 cells.

	Protein name	MASCOT Score	Predicted MW (Da)	Peptide no.
Cytoskeleton associated proteins	MARCKS-related protein	71	19,517	1
RNA binding proteins	Lupus La protein	107	21,429	1
Heat shock proteins	Heat shock protein HSP 90- α	523	84,607	15
	Heat shock protein HSP 90- β	490	83,212	15
Post-translational modifications	T-complex protein 1 subunit ϵ	223	55,314	3
	T-complex protein 1 subunit α	145	46,880	2
	Elongation factor 2	185	95,277	7
Transport proteins	Sodium/potassium-transporting ATPase subunit α	114	78,857	1
Clearance of lipids	Lactadherin	241	43,095	7
Signalling transducer proteins	14-3-3 protein ϵ	236	29,155	6
	14-3-3 protein θ	194	27,747	6
	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	95	45,575	2
DNA related proteins and transcription factors	X-ray repair cross-complementing protein 5	83	82,652	2
	X-ray repair cross-complementing protein 6	77	64,243	2
Glycolytic and TCA enzymes	Phosphoglycerate mutase 2	78	28,748	2
Ribosomal proteins	40S ribosomal protein S3	61	14,994	1
	40S ribosomal protein S8	58	21,866	1
	Ubiquitin-like modifier-activating enzyme 1	286	117,774	6
Other proteins	Brain acid soluble protein 1	82	22,680	2

was based on the quantification of nucleic acid and total protein content as well as the proteomic composition by nano-LC-MS/MS.

The results showed the presence of host cell DNA and RNA within the VLPs as well as host cell proteins (Table 1). The proteomic composition revealed that several host cell proteins were incorporated in influenza Gag-VLPs and co-produced EVs as well as in separately produced EVs. The identified proteins were multifunctional proteins mostly involved in cell signalling pathways, protein translocation and folding, cell cytoskeleton and nucleic acid binding. The RNA-binding protein nucleolin was only identified in “VLPs-EVs”, probably due to a specific interaction with the Gag protein. On the other hand, lactotransferrin was the most abundant protein in EVs, as well as the HSP90. In addition, important proteins associated to the endosomal sorting complexes required for transport (ESCRT) were identified in the “only EVs”, whereas they were not detected in “VLPs-EVs”.

The concentrations of DNA and RNA measured within these preparations underlined the need for further studies more focused on determining the nucleic acid composition and revealing what sort of information they carry. In addition, proteins, mainly membrane proteins, that were uniquely identified in VLPs-EVs can be exploited to develop novel affinity purification protocols to separate EVs from VLPs. This could be considered expecting that potentially those proteins, such as nucleolin, that were related to Gag protein are unique from VLPs and are not incorporated in the co-produced EVs, but this remains to be experimentally demonstrated. Furthermore, proteins that were uniquely identified in each preparation with a high MASCOT score might be overexpressed by engineering HEK-293 host cells to assess any potential benefit to improve VLPs or EVs expression.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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