Formation of virus-like particles from human cell lines exclusively expressing influenza neuraminidase.

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1   *Full title:* Formation of virus-like particles from human cell lines exclusively expressing

2   Influenza neuraminidase.

3

4   *Running title:* Influenza NA alone can form virus like particles.

5

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16

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21

22   **Summary:**

23   The minimal viral requirements for the generation of influenza virus like particle (VLP)

24   assembly and budding was reassessed. Using neuraminidase from the H5N1 and H1N1

25   subtypes, it was found that the expression of neuraminidase (NA) alone was sufficient to

26   generate and release VLPs. Biochemical and functional characterization of the NA containing

27   VLPs demonstrated that they were morphologically similar to influenza virions. The NA

28   oligomerization was comparable to that of the live virus, and the enzymatic activity, while not

29   required for the release of NA-VLP, was preserved. Together, these findings indicate that NA

30   plays a key role in virus budding and morphogenesis and demonstrates that NA-VLPs

31   represent a useful tool in influenza research.

32

33

34   **Introduction**

35   Influenza A viruses are lipid enveloped members of the *Orthomyxoviridae* family.

36   They contain 8 negative-sense, single-stranded RNA segments encoding 10 viral proteins.

37   Influenza virions are pleomorphic although generally their shape is roughly spherical with a

38   diameter around <150 nm. However, larger [100-400 nm] influenza virions are also

39   generated, as are filamentous forms (Fujiyoshi *et al.*, 1994).

40

41   Influenza viruses derive their lipid envelope by budding from the plasma membrane of

42   the infected cells and progeny virions are normally not found inside the host cell. Therefore

43   assembly and budding are the final, but essential steps in the virus life cycle. M1 matrix

44   protein is the most abundant protein in the influenza virion and plays a critical role in both

45   virus assembly and budding (reviewed in Nayak *et al.*, 2009). M1 affects virus assembly by

46   interacting with the core viral ribonucleocapsid (vRNP) and cytoplasmic tail of

47   transmembrane proteins, forming a bridge between the two layers, as well as recruiting the

48   internal viral proteins and vRNA to the plasma membrane in a cooperative manner (Noda *et*

49   *al.,* 2006). In addition, M1 interacts with the lipid bilayer producing an outward bending of the

50 membrane and this has been postulated to be the major driving force of influenza budding

51 since cells expressing M1 protein alone assemble into virus-like particles (VLPs) (Gómez-

52 Puertas *et al.,* 2000; Latham & Galaeza, 2001).

53

54   Haemagglutinin (HA) and neuraminidase (NA) are the two major surface

55 glycoproteins in the influenza viral membrane. HA binds to sialic acid receptors on the cell

56 surface and mediates the fusion process (Matrosovich *et al.,* 2006); whereas NA cleaves the

57 terminal sialic acids from the cell surface glycans to facilitate release of the progeny virus

58 from the host cell and prevent aggregation of virus particles (Bucher & Palese, 1975; Air &

59 Laver, 1989). NA is also essential in the initial stage of virus infection by enhancing HA-

60 mediated fusion (Su *et al.*, 2009), helping the virus to penetrate the mucin barrier protecting

61 the airway epithelium (Matrosovich *et al.,* 2004) and promoting virus entry (Ohuchi *et al.*,

62 2006). Studies of mutant influenza viruses have previously shown that the cytoplasmic tails

63 of HA and NA contribute to control virus assembly (Zhang *et al.*, 2000) and virus morphology

64 (Jin *et al.,* 1997). The specific role of HA and NA in virus budding is controversial. COS-1

65 cells expressing HA alone did not give rise to virus like particles (VLP) production (Gómez-

66 Puertas *et al.,* 2000), however assembly and budding of a NA-deficient virus mutant could be

67 rescued with exogenous bacterial NA to the level of wild-type virus (Liu *et al.,* 1995). These

68 studies suggested that the presence of both viral proteins was not essential for virus budding.

69 Nevertheless, studies of cells expressing recombinant viral proteins from an H3N2 virus

70 demonstrated that both HA and NA were involved in the budding process (Chen *et al.,* 2007).

71

72   In this study, using a plasmid-driven VLP production system in human embryonic

73 kidney (HEK-293T) cells, we looked at the minimal viral requirements for influenza virus

74 assembly and budding. We showed that both H5 HA and N1 NA can be the driving forces for

75 virus budding, whereas M1 only had a limited contribution. In addition, we demonstrated that

76 expression of NA alone could lead to the budding of particles. The influenza VLPs with NA

77 (NA-VLPs) were morphologically similar to influenza virions and had an intact sialidase

78 enzymatic activity. Comparable results in generating NA VLPs were found with NA from both

79 seasonal and pandemic H1N1 and avian H5N1 viruses.

80

81 **Results**

82   **Determination of minimal viral requirement for VLP formation and release.** In

83 order to determine which viral structural protein(s) is/are essential for VLP-formation and

84 release, HEK-293T cells were transfected with plasmids encoding for H5N1 proteins HA, NA

85 and M1 singly, or in combination. Protein composition of the VLPs released into the culture

86 medium was analyzed by western blotting (Fig.1a) and budding of VLPs from the transfected

87 cells was visualized by transmission electron microscopy (Fig. 2). None of the transfections

88 affected the protein expression system as indicated by analysis of the glyceraldehyde 3-

89 phosphate dehydrogenase (GAPDH) housekeeping protein expression. Co-transfection of

90 genes coding for different viral proteins had a minimal impact on individual expression level

91 as shown in cell lysates (Fig. 1a). The expression of the HA, NA and M1 in the cytosol

92 appeared to be comparable. Release was quantified by densitometric analysis of the

93 Western blots and expressed as a percentage of overall production (cumulative both from

94 lysates and in supernatant). VLPs were not detected by western blot from the culture

95 medium of cells expressing HA alone unless exogenous bacterial NA was added (Fig. 1a,

96 lanes 1 vs. 2), suggesting that VLPs budding from HA-expressing cells could not be released

97 without exogenous NA as confirmed by VLP aggregation on the cell surface in TEM (Fig. 2a

98 vs. 2c). When M1 was expressed alone, a small amount of the protein was detected in the

99 supernatant and no significant change was observed by addition of exogenous NA (Fig. 1a,

100 lanes 4 & 5). However, we were not able to find VLP structures from cells expressing M1

101 alone (Fig. 2e) When HA and M1 were co-expressed, VLPs were released into the

102 supernatant only in the presence of exogenous bacterial NA (Fig. 1a, lanes 7 & 8) with an

103 increase of HA and M1 content when compared to their single-expression. Interestingly,

104 release of both HA and M1 was significantly enhanced by the co-expression of NA (Fig. 1a,

105 lanes 3, 6 & 9). Also, VLPs containing NA were easily detected from the cells expressing NA

106 exclusively (Fig. 1a, lane 10) and could be widely observed by TEM (Fig. 2d & 2f). These

107 results confirmed the requirement of the NA enzymatic activity for the HA-containing VLP

108 release, but also suggested a role of NA in VLP budding. Comparison of protein expression

109 in the cell lysates as well as in the supernatant of infected or HA/NA/M1 transfected cells

110 showed that HA and NA had similar expression levels (Fig. 1b), however the amount of M1

111 released with the budding VLPs in the supernatant was approximately half of that found in

112 virus infected cells. This was compatible with previous reports that M1 requires interaction

113 with other viral proteins, such as the structural proteins HA/NA (Wang et al., 2010), as well

114 as with viral RNPs which are absent from VLPs. Using TEM, the VLPs were pleomorphic

115 with spherical or filamentous forms (Fig. 2b & 2f), similar to influenza virions.

116

117 **Physical and functional characterization of NA-VLPs produced in cells**

118 **expressing influenza virus NA alone.** NA from the H5N1 subtype and from seasonal H1N1

119 and pandemic H1N1 subtypes were included in this study. Culture medium of cells

120 expressing the NA protein alone was harvested for detection of released particles. FLAG-

121 tagged NAs were used to allow the detection with anti-FLAG antibodies of the protein from

122 different viral origins at the same antibody binding affinity. No difference of VLP formation

123 efficiency was observed between tagged and non-tagged NA (data not shown). Furthermore,

124 similar particles were seen by TEM for all three subtypes we studied (Supplementary data

125 S1). Sucrose gradient (20-60 %) centrifugation analysis demonstrated the presence of NA-

126 VLPs in the intermediate pellet fractions between 30-50 % sucrose (Fig. 3 b-d), which was

127 similar to the result observed with influenza A/WSN/33(H1N1) virions (Fig. 3a). In contrast,

128 purified NA proteins were only present in lighter fractions (~20 % sucrose) of the gradient

129 (Fig. 3e). In addition, NA proteins either purified or on the VLP surface or from the virus were

130 functionally active as shown using a neuraminidase activity assay (Fig. 3f). The apparent

131 shift of one fraction towards a higher concentration of sucrose for the virus versus the VLP

132 was probably due to the presence of nucleic acid in the virus (RNPs) decreasing slightly their

133 buoyancy.

134

Kinetic analysis of the NA-VLP production showed that it was continuous over at least
60 hours post-transfection with continuous accumulation of the NA-VLP in the supernatant
(Fig. 4a) when using NA from both pandemic H1N1 or from H5N1. Cleavage of caspase-3
was not detected in all time-points indicating there was no evidence of apoptosis (Fig. 4b).
Electron micrographs of transfected cells also failed to manifest features of apoptosis during
the course of the experiment.

141

We found that sole expression of NA (from H5N1) was sufficient to promote release of
NA-VLP (Fig. 5). A mutation of NA in position 262 (E to D) which abrogated the sialidase
activity as well as treatment with Oseltamivir (sialidase inhibitor) failed to affect VLP
formation. As expected, co-expression of HA (H5 from H5N1) increased the release of NA
probably by the formation of heterochimeric VLP (HA/NA-VLP), but release of HA-containing
VLP was then dependent on sialidase activity.

148

Immunoblotting analysis of VLPs showed that the NAs on the particle surface were in
multimeric form, including the monomer (~55 kDa), dimer (~110 kDa), trimer (~165 kDa) and
tetramer (~220 kDa) (Fig. 6). Multimeric NA complexes on the VLPs were denatured into
monomer by heating in SDS loading buffer with chaotropic agent (urea) or reducing agent
(DTT) (lanes 1 to 3). Upon pre-treatment of DTSSP before the denaturing step, multimeric
NA complexes were protected from SDS and urea, and partially from DTT (lanes 4 to 6).
Similar multimeric NA complexes were detected in the H1N1 virions but the detection failed
in the samples pre-treated with DTSSP (lanes 7 & 8), probably due to the inability of the
antibody to recognize cross-linked antigens. Samples from highly pathogenic H5N1 virus
could not be analyzed as protocols for inactivating the infectivity of the highly pathogenic
virus H5N1 so that these preparations could be taken out of bio-safety level-3 containment
for further analysis compromises oligomer integrity.

161

162   Desialylation of cell surface sialic acids in cells expressing NA was then studied by

163   lectin binding (Supplementary data S2). A weak SNA binding was observed in HEK-293T

164   cells, indicating low level of α-2,6 linked sialic acid on the 293T cells, and this disappeared in

165   cells expressing any of the NAs studied. Similar loss of cell-surface sialic acids was seen in

166   NA VLP expressing A549 or MDCK cells which express more α-2,6 sialic acid (data not

167   shown). MAA binding confirmed the presence of α-2,3 linked sialic acid on mock transfected

168   293T cells and this was significantly decreased in NA-expressing cells. Cleavage of sialic

169   acids by sialidase is expected to expose the underlying Gal-GalNAc and this was

170   demonstrated by the increased binding of PNA to NA-expressing cells.

171

172   **Discussion**

173   Influenza assembly, budding and release are the last but important steps in the

174   replication cycle. VLP production assays using different systems have been developed to

175   determine the viral proteins involved in the virus budding (Gómez-Puertas *et al.,* 2000;

176   Latham & Galaeza, 2001; Neumann *et al.,* 2000). Here we investigated the minimal viral

177   components required for assembly and budding of H5N1 influenza virus using a plasmid-

178   driven VLPs formation system similar to that described in a previous study on H3N2 virus

179   (Chen *et al.*, 2007).

180

181   Previous findings about the major viral proteins responsible for the virus budding have

182   been contradictory. It was suggested that M1 plays the major role in driving virus budding

183   from the cellular membrane (Gómez-Puertas *et al.,* 2000), whereas Chen and his colleagues

184   found that M1 is not essential for the process but that HA and NA were necessary (Chen *et*

185   *al.,* 2007). A recent publication (Wang et al., 2010) confirmed that M1 by itself fails to form

186   virus-like particles, probably due to a lack of membrane-targeting signal and therefore

187   requires the interaction with other viral proteins to be incorporated in the budding virions. In

188   our study, although small amounts of M1 were released from the cells expressing M1 alone,

189   no budding particle was observed by electron microscopy (Fig. 2e). A similar finding was

190   recently reported in which it was suggested that M1 was only nonspecifically secreted into

191   the supernatant rather than released in the form of VLP (Tscherne et al., 2010). Nevertheless,

192   co-expression of M1 increased the level of both HA and NA incorporated into the VLPs

193   (compare Fig. 1a, lanes 2 & 8; lanes 6 & 10), suggesting that M1 helps the virus budding,

194   probably by pushing the inner side of the cellular membrane.

195

196   VLP formation in cells transfected with HA alone or HA/M1 was completely dependent

197   on the addition of exogenous NA. In the absence of exogenous NA, HA-containing VLPs

198   were aggregated on the cell surface and not released into the medium (Fig. 1 & 2).

199   Exogenous NA cleaved the cell surface sialic acids allowing the VLPs to be released,

200   therefore both HA and M1 became detectable in the medium. These results suggested that

201   the expression of HA could provide a driving force to trigger the production of VLP, although

202   exogenous NA was necessary for the release of the particles.

203

204   Previously it has been suggested that NA was important for virus morphogenesis.

205   Studies using influenza virus lacking cytoplasmic tails of HA or NA (HAt- /NAt-) demonstrated

206   that NAt- virus particles had an elongated morphology but not HAt- virus (Jin *et al.,* 1997).

207   Introduction of mutations in both the transmembrane and cytoplasmic domains of NA

208   confirmed that NA is critical to control the virus shape, size and titer (Barman *et al.,*

209   2004).Studies of mutant virus containing a large internal deletion in NA gene have shown

210   they can assemble and bud similarly to wild-type virus (Liu *et al.,* 1995). However, our data

211   has shown for the first time that expression of NA alone in HEK-293T cells could provide a

212   driving force for the formation of extracellular NA-containing particles and this finding was

213   seen with NA from different influenza viruses of the N1 subtype and demonstrated in at least

214   two additional human cell-lines (A549 and HeLa, data not shown). It is also important to note

215   that the release of M1 and HA was greatly enhanced by the co-expression with NA to a level

216   which was not acquired by high concentration of exogenous NA, indicating that NA is likely to

217   be a major force in driving virus budding. However, whereas Chen et al. (2007) reported that

218 only small amounts of NA were released when this glycoprotein was expressed on its own,

219 our data showed that a large amount of NA was released. This discrepancy is probably due

220 to the difference in influenza virus subtype used in the two studies (N2 in Chen et al., versus

221 N1 here). In fact we did observe that N2 subtype NA was expressed to much lower level than

222 N1 and that N1 from human seasonal H1N1 was expressed less than the highly pathogenic

223 avian H5N1 (Supplementary data S3).

224

225   Furthermore, using a potent sialidase inhibitor and a point mutation (E262D) in NA

226 that inactivates the catalytic site of the sialidase (Huang et al., 2008), we could show that

227 neuraminidase activity of the NA was not necessary for the release of NA-VLP in cells (Fig.

228 5).

229

230   To characterize the NA-VLPs, biochemical, physical, morphological and functional

231 studies were done, including sucrose gradient flotation profile, NA functional assay, detection

232 of multimeric NA, TEM study, as well as desialylation of surface sialic acid on the cells

233 producing NA-VLPs. The presence of both influenza virions and NA-VLPs in the middle

234 fractions of the sucrose gradient (Fig. 3a and b-d) indicates that VLPs have a buoyant

235 density equivalent to native influenza viruses. The enzymatic activity assay showed that NAs

236 on the particle surface were functionally active and the activity was in proportion to the

237 amount of protein (Fig. 3f). The morphology of the VLPs was found to resemble influenza

238 virions. Most of the VLP were spherical although elongated particles were also detected (Fig.

239 2b & 2f). No morphological difference was observed in NA-VLPs from high pathogenic avian

240 influenza H5N1, seasonal H1N1, and pandemic swine-origin H1N1 (Supplementary data S1).

241

242   It is conceivable that exosomes or vesicles arising from apoptotic bodies may be

243 mistaken as VLPs. However, electron microscopy failed to reveal either apoptosis or the

244 formation of intracellular vesicles (such as the multivesicular bodies of exosomes).

245 Furthermore, we also monitored for apoptosis in transfected cells by attempting to

246   demonstrate the expression and cleavage of caspase-3 in the course of the experiment. Only

247   the uncleaved form of caspase-3 could be detected for the first 60 hours post-transfection

248   suggesting that VLP-producer cells were not undergoing apoptosis (Fig. 4b). Cross-sections

249   of membrane protrusions such as filopodia (Fig. 2a, arrowhead B) do occur and these may

250   be mistaken as budding of VLP. However, they can be distinguished from the "typical VLP"

251   by electron microscopy because VLPs (Fig. 2a, arrowhead A) have a more electron dense

252   outline which is distinguishable from the cell membrane, except at the budding sites of virus

253   or VLPs (Fig. 2). Taken together, these data indicate that the particles observed in the EM

254   are true viral like particles.

255

256   NAs on the particle surface were found to be tetrameric in the influenza virions, which

257   is important for some of the NA functions. Using the cross-linker DTSSP, multimeric NA were

258   protected from urea and we were able to detect NA multimeric complexes comparable to that

259   found on fully infectious H1N1 virions. This result was expected as maturation of the NA has

260   been described to take place in the endoplasmic reticulum (ER) where the NA-tetramers

261   form before going through the Golgi network to the assembly site at the plasma membrane

262   (Saito et al., 1995). Therefore, budding of the NA-VLPs (or virus) from the cell surface will be

263   expected to incorporate only mature tetrameric NA proteins with similar oligomerization as

264   would the influenza virus. Failure to detect NA from virions after DTSSP treatment may be

265   caused by conformational changes of the protein upon conjugation. The cross-linker may

266   also modify or hide the residues (such as lysine) involved in the antibody binding site. Our

267   data indicated that NA on the VLP surface was in oligomeric form although the technique

268   used did not allow us to quantify the ratio between the monomer, dimer, trimer and tetramer

269   (Fig. 6).

270

271   All three neuraminidases included in the study were able to cleave cell surface sialic

272   acids in both α-2,3 and α-2,6 linkage. As the technique used is qualitative rather than

273   quantitative, we could not quantify a preference in the cleavage activity to sialic acid with α-

274   2,3 linkage (Supplementary data S2), as mentioned in previous findings using H1N1 and

275   H3N2 virus NA (Mochalova *et al.*, 2007; Franca de Barros *et al.*, 2003). We detected a

276   significant increase of β-Gal-GalNAc in NA-expressing cells by the PNA binding. This

277   supports the role of NA enzymatic activity during the initial stage of infection by helping the

278   virus to penetrate through the mucus layer where sialic acid is mainly linked to Gal-GalNAc in

279   α-2,3 configuration.

280

281   Neuraminidase is an important viral component of influenza viruses and the most

282   effective anti-influenza drugs in the market are NA inhibitors. Although NA has been studied

283   for decades, most of the data were obtained from purified virion which requires access to a

284   high bio-containment laboratory when dealing with highly pathogenic avian influenza viruses.

285   Purified NA proteins were also widely used for NA study but these isolated NA may not have

286   the same properties as NA on viral surface. It was in fact reported that substrate specificities

287   of sialidase activity from purified NA and their original viruses were not identical (Nagai *et al.*,

288   1995). NA-VLPs produced in our study, though non-infectious, were multivalent, antigenic,

289   enzymatically active and morphologically similar to native influenza viruses. Therefore they

290   could be a preferable alternative to recombinant NA peptides or proteins as immunogen to

291   produce antibodies against neuraminidase or as antigens for ELISA type assays. In addition,

292   because NAs on the VLPs surface were not only functionally active but also presented in

293   multimeric form, these VLPs are a useful tool to investigate NA interactions with the host

294   cells, as well as neuraminidase inhibitor drugs.

295

296   **Methods**

297   **Plasmids.** Plasmids pcDNA-HA, pcDNA-NA(H5), and pcDNA-M1 respectively

298   corresponding to DNA sequences of HA (GI:126361929), NA (GI: 126361907) from influenza

299   A/Cambodia/JP52a/2005 (H5N1) and M1 (GI: 81975893) from influenza

300   A/Goose/Guangdong/1/96 (H5N1) were cloned into the mammalian expression vector

301   pcDNA3.1 (Invitrogen). Both non-tagged and FLAG-tagged HA and NA proteins in the C-

terminus were used. For the study of neuraminidase from different viral subtypes, plasmids pcDNA-NA(H1) and pcDNA-NA(pdmH1) encoding the FLAG-tagged NA of influenza A/Gansu/Chenguan/1129/2007 (seasonal H1N1; GI:194140557) and A/California/04/2009 (swine origin pandemic H1N1; GI:227977118) were prepared respectively. Enzymatically inactive pcDNA-NA(E262D) was prepared from pcDNA-NA(H5) by point mutation using QuikChange® II Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions and post-sequencing selection of clones based on NA-Star chemoluminescent assay (Applied Biosystems).

Cells and viruses. HEK-293T and MDCK cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin at 37 °C with 5 % CO_2. Influenza A/WSN/33 (H1N1) virus was propagated in MDCK cells with culture medium supplemented with 0.5 μg.mL^{-1} TPCK-treated trypsin. Highly pathogenic avian influenza (HPAI) A/Hong Kong/483/97 H5N1 virus was propagated in MDCK in MEM culture medium.

Antibodies and Western blotting. Vaccinated chicken sera containing anti-HA antibodies (haemagglutination inhibition titer >256) and polyclonal antibodies from rabbit hyper-immunized with avian Influenza A neuraminidase (Abcam) were used to detect non-tagged HA and NA proteins respectively. Anti-FLAG monoclonal mouse antibody (Sigma) was used to detect FLAG-tagged HA and NA. Monoclonal mouse antibody GA2B (Abcam) was used for the detection of influenza M1 protein. For western blotting, samples were resolved in 4-12 % SDS-PAGE (Invitrogen), electroblotted to polyvinylidene fluoride transfer membrane, hybridized with appropriate antibodies and detected with ECL Western Detection Reagent (Amersham). Protein size was estimated using the Novex® Sharp Pre-stained Protein Standard (Invitrogen). The fraction of protein released in supernatant (SN) was expressed as a percentage of overall expressed protein both in lysates and in SN adjusted to the respective volumes followed by normalization relative to GAPDH

330   housekeeping protein expression detected with anti-GAPDH Mouse Monoclonal (6C5)

331   antibody (Abcam). Quantification was performed by densitometry on scanned images

332   obtained using Scion Image Beta 4.0.3 (Scion Corporation). Each quantification was

333   associated with a calibration curve using a range of BAP-flag protein (Sigma) covering the

334   range of concentration measured. The exposure times were chosen in order to quantify the

335   signal intensity within the linear range of the calibration curve below saturation of the signal

336   as monitored by the image analysis.

337

338   **Detection of VLP production in cells expressing influenza HA, NA and M1**

339   **proteins.** HEK-293T cells were transfected with different combinations of plasmids using

340   CalPhos Mammalian Transfection Kit (Clontech) according to the manufacturer's instructions.

341   The amount of plasmids pcDNA-HA, pcDNA-NA(H5) and pcDNA-M1 in the transfection

342   mixture was 5 μg each, and empty vector was used to replace the omitted plasmids when

343   appropriate. At 12 hours post-transfection, the medium was replaced with fresh DMEM-10%

344   FBS, and exogenous bacterial neuraminidase (*Vibrio cholerae*, Roche; 6.25 mU.mL^{-1}) was

345   added to the medium when appropriate. At 60 hours post-transfection, supernatant was

346   collected and cell debris were removed by filtration through 0.45 μm filters. Filtered culture

347   medium was then layered onto a 30 % sucrose-HEPES buffer (2 mM HEPES, 125 mM NaCl,

348   0.9 mM CaCl$_2$, 0.5 mM MgCl$_2$, pH 7.4) cushion and centrifuged at 28,000 rpm for 2.5 hours at

349   4 $^\circ$C and the pellet was resuspended in HEPES buffer. Cell lysates were collected at the

350   same time by the addition of lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 0.1 %

351   SDS, 1 % Triton-X, protease inhibitor mixture (Roche Diagnostics) followed by centrifugation

352   at 13,000 rpm for 10 minutes. Samples were mixed with 4X LDS sample buffer (Invitrogen)

353   and dithiothreitol (DTT, 100 mM), boiled for 8 minutes and analyzed by western blotting as

354   described above.

355

356   **H5N1 virion preparation and analysis.** MDCK cells, plated the day before, were

357   inoculated for 1 h at 37 °C with H5N1 virus at multiplicity of infection of 1. After washing with

358   phosphate buffer saline (PBS), infected cells were incubated at 37 °C overnight. Twenty

359   hours post-infection, the supernatant was harvested, clarified by centrifugation before

360   concentration on 100 kDa pore filter Amicon system (Millipore) and finally resuspended in

361   lysis buffer (50 mM KCl, 1 % NP-40, 25 mM HEPES (pH 7.4), 1 mM DTT, protease inhibitor

362   mixture (Roche Diagnostics)). Cell lysates were also collected after addition of lysis buffer to

363   the infected cells. In each sample, loading buffer (prepared from 6X: 62.5 mM Tris-HCl (pH

364   6.8), 25 % Glycerol, 2 % SDS, 0.01 % bromophenol blue, 5 % β-mercaptoethanol) was

365   added, boiled for 10 minutes so they can be taken out from the biosafety level 3 laboratory

366   (BSL-3).

367

368   **NA-VLP and H1N1 virion preparation and analysis.** For NA-VLP production, HEK-

369   293T, HeLa, and A549 cells were transfected with 10 µg of pcDNA-NA(H5), pcDNA-NA(H1)

370   or pcDNA-NA(pdmH1) as indicated above. Transfection with equal amount of empty vector

371   was carried out as a mock control. After ultracentrifugation of clarified supernatant (as

372   described above), the pellet was resuspended in NTE (100 mM NaCl, 10 mM Tris (pH 7.4), 1

373   mM EDTA), put onto a sucrose gradient (20-60 %) and centrifuged at 35,000 rpm for 2 hours

374   at 4 °C. Twenty fractions were manually collected from the top to bottom.

375

376   Influenza A/WSN/33 (H1N1) virions were purified from infected MDCK cultures at 72

377   hours post-infection. The supernatant was harvested and clarified by centrifugation at 4,000

378   rpm for 15 min. Virions were then pelleted, put onto sucrose gradient (20-60 %) and

379   centrifuged as described above for VLPs. Recombinant NA proteins purified from transfected

380   cell lysates by immunoprecipitation using anti-FLAG M2-agarose affinity gel (Sigma), were

381   layered onto sucrose gradient as above, as additional control.

382

383   All the upper-mentioned sucrose fractions of VLPs, virions and purified proteins were

384   boiled in LDS loading buffer with DTT, and analyzed by western blotting (see above).

385   Sialidase activities of the NA-VLPs and purified proteins were measured by NA-Star

386 chemoluminescent assay (Applied Biosystems). Oligomerization of NA on the VLP surface

387 was studied using the cross-linker 3,3´-dithio-bis(sulfosuccinimidylpropionate) (DTSSP,

388 Thermo Scientific). NA-VLPs were mixed with DTSSP (5 mM) to stabilize the multimeric NA

389 complexes, prior to the addition of LDS loading buffer followed by addition of urea (8 M) or

390 DTT (100 mM) when appropriate. DTSSP pre-treated VLPs were directly analyzed by

391 western blotting as described above.

392

393 **Electron microscopy (EM).** Transfected HEK-293T cells were fixed with 2.5 %

394 glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and post-fixed with 1 % osmium tetroxide.

395 The fixed cells were then pelleted in 1 % agar, dehydrated in increasing concentration of

396 ethanol, and embedded in epoxy resin. Ultra-thin sections of the samples were visualized

397 with Philips EM208s electron microscope after staining with 2 % aqueous uranyl acetate and

398 Reynold's lead citrate.

399

400 **Kinetics of NA-VLP release.** HEK-293T cells were transfected with pcDNA-NA(H5)

401 or pcDNA-NA(pdmH1) as mentioned above. At 12 hours post-transfection, the culture

402 medium was replaced with fresh DMEM-10% FBS. Supernatant and cell lysates were

403 collected at 20, 28, 36 and 60 hours post-transfection as described above. Samples were

404 boiled with LDS loading buffer and DTT for 8 minutes and were analyzed by western blotting.

405

406 **Effect of neuraminidase enzymatic activities on production of VLP.** A single

407 amino acid change E262D in the NA is known to abrogate the sialidase activity of the NA

408 molecule (Huang et al., 2008). HEK-293T cells were transfected with pcDNA-NA(H5) or

409 pcDNA-NA(E262D), with or without pcDNA-HA. At 12 hours post-transfection, the culture

410 media were refreshed and sialidase inhibitor (Oseltamivir carboxylate, 50 μM) was added to

411 the medium as indicated. Supernatant and cell lysates were collected at 60 hours post-

412 transfection and protein levels in VLPs were analyzed by western blotting. Sialidase activities

413 of the supernatant were measured by NA-Star chemoluminescent assay (Applied

414 Biosystems).

415

416 **Lectin staining for cells surface sialic acid.** Lectin histochemistry analysis was

417 performed as previously described (Nicholls *et al., 2007*). Briefly, HEK-293T cell lines stably

418 or transiently expressing N1 from seasonal H1N1 (HuN1) or pandemic H1N1 (pdmN1) or

419 avian influenza H5N1 (AvN1) were fixed with 4 % paraformaldehyde (PFA). After washing

420 with PBS, lectin staining was performed with *Sambucus Nigra* agglutinin (SNA-I, Roche) and

421 *Maackia amurensis* agglutinin (MAA-II, Vector) to detect α-2,6 or α-2,3 linked sialic acid (SA)

422 respectively. Peanut agglutinin (PNA, Roche) was used to identify the terminal Gal-GalNAc.

423 Biotin conjugated lectins were used with avidin-peroxidase counterstaining (Vector).

424

432

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526

527   **Figures legends:**

528

529   **Figure 1. Detection of viral proteins from transfected HEK-293T cells.** (a) Evaluation of

530   the contribution of different viral structural proteins HA, NA, and M1 from H5N1 expressed in

531   HEK-293T cells in combinations as indicated and comparison with H5N1 whole virus (b).

532   Exogenous bacterial NA (exoNA) was added to the medium when indicated. VLPs released

533   in the culture medium were harvested at 60 hr post-transfection and pelleted through a 30 %

534   sucrose cushion. Samples were analyzed by SDS-PAGE followed by immunoblotting to

535   detect different viral proteins together with the lysates of transfected cells. Percentages of

536   protein released were calculated as fraction detected in the supernatant from the overall

537   protein detected both in lysates (Lys) and supernatants (SN). Apparent sizes of the HA, M1

538   and NA were ~27 kDa (HA2), ~28 kDa and ~55 kDa respectively.

539

540   **Figure 2. Transmission Electron Microscopy of cells expressing HA, NA and M1 from**

541   **A/Cambodia/JP52a/2005 (H5N1).** HEK-293T cells were processed for electron microscopy

542   at 36 h post-transfection. VLP budding was observed in cell expressing HA without

543   exogenous NA treatment (a & b) or with the addition of exogenous NA (c) or when NA was

544   expressed alone (d & f), but not when M1 was expressed alone. (e). Arrowheads : A: VLP; B:

545   filopodia.

546

547   **Figure 3. Physical and functional characterization of NA-VLPs.** Western blot analysis of

548   fractions from sucrose gradient from 20 % (fraction 1) to 60 % (fraction 20) sucrose of

549   A/WSN/33 (H1N1) virions collected from infected MDCK cells (a), from supernatant of HEK-

550   293T cells transfected with NA plasmid from seasonal H1N1 (b), pandemic H1N1 (c), HPAI

551   H5N1 (d) or from immuno-purified N1 protein (e). Neuraminidase enzymatic activity of the

552   corresponding sucrose fractions were tested with NA-Star chemoluminescent assay (f) and

553   expressed in arbitrary unit (AU) defined as the percentage of the fraction with the highest

554   signal.

555

Figure 4. Kinetics of N1-VLP release. (a) Neuraminidase enzymatic activity (expressed in relative luminescence unit, RLU) from the supernatant of HEK-293T cells transfected with NA from either pandemic H1N1 (plain line) or H5N1 (broken line) tested with NA-Star chemoluminescent assay. Experiment performed in triplicate. (b) Evaluation of percentage of NA released by western blot analysis of supernatant and transfected cell lysates; and monitoring of Caspase-3 cleavage (uncleaved (#) and cleaved (*) forms). The percentage of release was calculated as indicated in Methods section. Lysate from H5N1 virus-infected cells was used as positive control (Ctl) for cleaved form of caspase-3.

564

Figure 5. Influence of NA sialidase activity on NA-VLP release. Neuraminidase enzymatic activity (expressed in relative luminescence unit, RLU) from the supernatant of HEK-293T cells transfected with NA (either from H5N1 wild-type (wt), or with mutation E262D (mut)) alone or co-expressed with HA; in absence or presence of Oseltamivir (Oslt). Evaluation of percentage of NA released by western blot analysis of supernatant (SN) and transfected cell lysates (Lys) (2X). Percentage of NA release was calculated as indicated in Methods section. Bkg: background level.

572

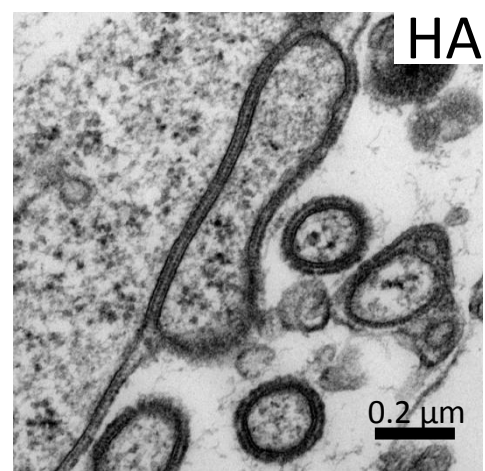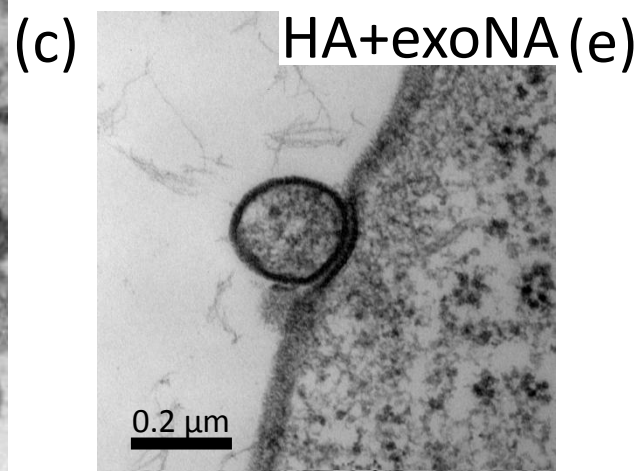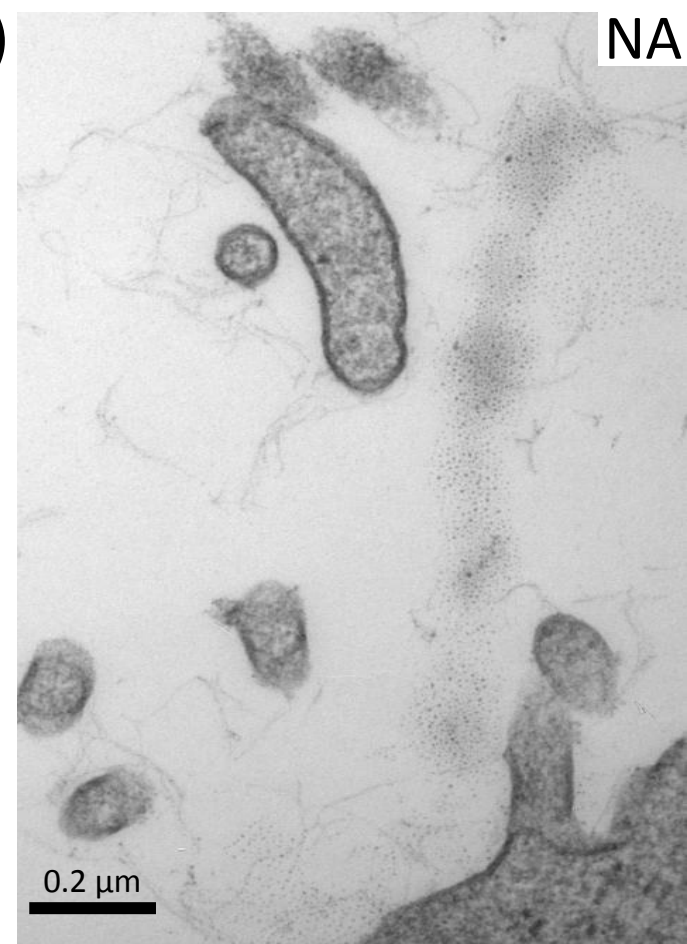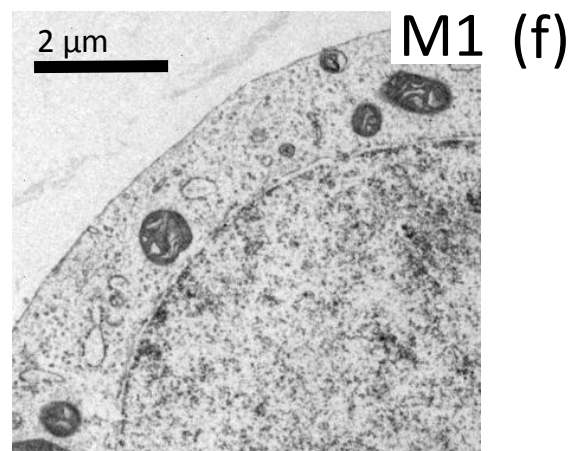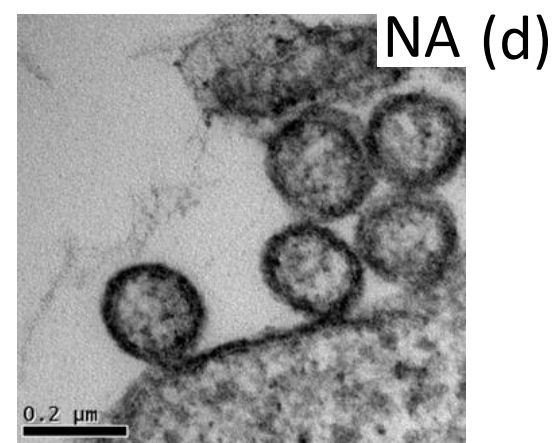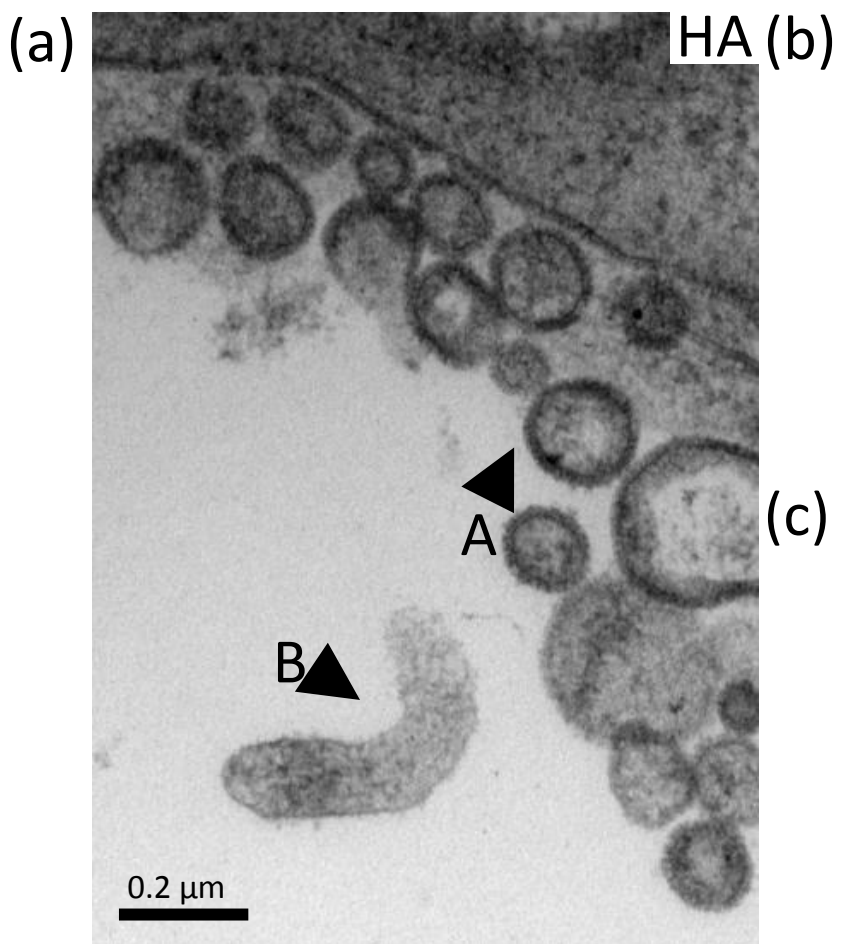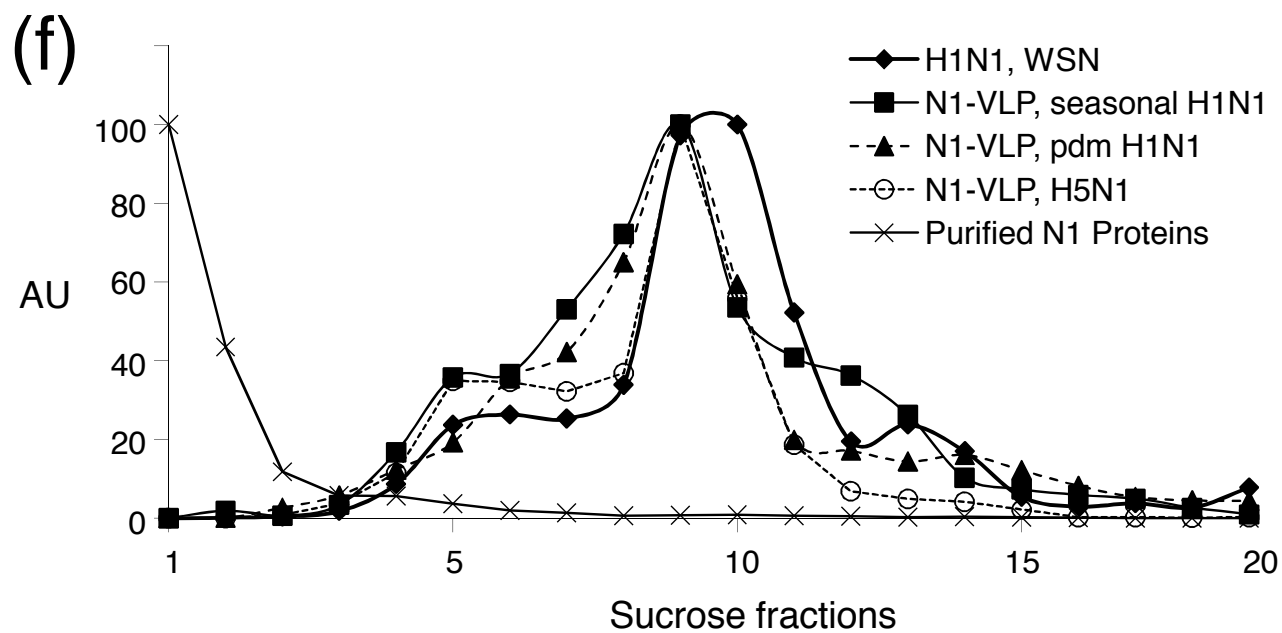**Figure 6. Detection of multimeric NA complexes on VLPs.** N1-VLPs were resolved in 4-12 % SDS-PAGE after heating in SDS loading buffer (lane 1), and with the addition of urea or DTT (lanes 2 and 3 respectively). Similar treatments were done on NA-VLPs pre-treated with cross-linker DTSSP (lanes 4 to 6). Samples from SDS-PAGE were then transfer to PVDF membrane and immunoblotted with monoclonal anti-FLAG antibodies. H1N1 virions were included for comparison and NA were detected using anti-NA antibodies. Apparent sizes of NA monomer, dimer, trimer and tetramer were ~55 kDa, ~110 kDa, ~165 kDa, and ~220 kDa respectively.

581

582

(a)

Released

α-M1

			4%	3%	33%		5%	31%		
SN
Lys.

α-HA

| | 2% | 33% | 49% | | | 3% | 36% | 50% | | |
SN
Lys.

α-NA

| | | 49% | | | 56% | | | 60% | 44% | |
SN
Lys.

GAPDH Lys.

	1	2	3	4	5	6	7	8	9	10	11
HA	+	+	+	−	−	−	+	+	+	−	−
M1	−	−	−	+	+	+	+	+	+	−	−
NA	−	−	+	−	−	+	−	−	+	+	−
exoNA	−	+	−	−	+	−	−	+	−	−	−

(b)

Released

	53%	27%
α-M1		
α-HA	56%	41%
α-NA	46%	46%
GAPDH		
	Lys. SN	Lys. SN
	H5N1 virus	HA/NA/M1 VLP

(a) HA

A

B

0.2 μm

(b) NA

0.2 μm

(c) HA+exoNA

0.2 μm

(d) M1

2 μm

(e) HA

0.2 μm

(f) NA

0.2 μm

20% ⟶ Sucrose Gradient ⟶ 60 %

(a)

(b)

(c)

(d)

(e)

(f)

AU

Sucrose fractions

— ♦ — H1N1, WSN
— ■ — N1-VLP, seasonal H1N1
– ▲ – N1-VLP, pdm H1N1
– ○ – N1-VLP, H5N1
— × — Purified N1 Proteins

(a)

(b)

Released 9% 16% 24% 36%

N1(H5) VLP

SN

Caspase-3

Ctl

\#

*

Lys.

23% 30% 38% 45%

N1(pdmH1) VLP

SN

Caspase-3

Ctl

\#

*

Lys.

Time post-transfection   20h   28h   36h   60h

SDS	+	+	+	+	+	+	+	+
Urea	−	+	−	−	+	−	−	−
DTT	−	−	+	−	−	+	−	−
DTSSP	−	−	−	+	+	+	−	+

Tetramer
Trimer

Dimer

Monomer

1	2	3	4	5	6	7	8

NA-VLP   virus