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Muzajjad Gozal Goffar[1], Vipin Kumar Deo[2], Tatsuya Kato[1,3], and Enoch Y. Park[1,3*]

[1] *Laboratory of Biotechnology, Faculty of Applied Biological Chemistry, Department of Agriculture, Shizuoka University. 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan.*

[2] *Organization for International Collaboration Shizuoka University. 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan.*

[3] *Laboratory of Biotechnology, Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan.*

[*] Corresponding author: *Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan.*

E-mail addresses: ozzagozal@gmail.com (M.G. Goffar), deo.vipin.kumar@cii.shizuoka.ac.jp (V.K. Deo), kato.tatsuya@shizuoka.ac.jp (T. Kato), park.enoch@shizuoka.ac.jp (E.Y. Park).

ABSTRACT

Rous sarcoma virus-like particles (RSV-LPs) displaying hemagglutinins of H1N1 (A/New Caledonia/20/99) (H1) and H5N1 (A/Vietnam/1194/2004) (H5) of the influenza A virus were produced. The H1 has its transmembrane domain, but the H5 was fused with the transmembrane domain of glycoprotein 64 (BmGP64) from *Bombyx mori* nucleopolyhedrovirus (BmNPV). H1 and RSV Gag protein were coexpressed in the hemolymph of silkworm larvae, copurified, and confirmed RSV-LP displaying H1 (VLP/H1). Similarly, the RSV-LP displaying H5 (VLP/H5) production was also achieved. Using fetuin agarose column chromatography, RSV Gag protein-coexpressed H1 and H5 in silkworms were copurified from the hemolymph. By immuno-TEM, H1 and H5 were observed on the surface of an RSV-LP, indicating the formation of bivalent RSV-LP displaying two HAs (VLP/BivHA) in the hemolymph of silkworm larvae. VLP/H1 induced the hemagglutination of red blood cells (RBCs) of chicken and rabbit but not sheep, while VLP/H5 induced the hemagglutination of RBCs of chicken and sheep but not rabbit. Additionally, VLP/BivHA allowed the hemagglutination of RBCs of all three animals. Silkworm larvae can produce RSV-LPs displaying two HAs and is a promising tool to produce the bivalent enveloped VLPs for the vaccine platform.

Keywords: Silkworm, Rous sarcoma virus, Virus-like particle, Hemagglutinin, Display, Bivalent

1. Introduction

Virus-like particles (VLPs) are particles composed of only structural proteins of viruses and have almost the same morphology. However, unlike viruses, VLPs do not have any genetic materials; therefore, they do not have any infectivity to hosts [1]. Therefore, VLPs are regarded as promising tools for developing vaccines for infectious diseases and carriers of drug delivery systems [2].

VLPs have two types of structure, enveloped and non-enveloped [3], similar to viruses, which are also divided into enveloped and non-enveloped viruses. Non-enveloped VLPs are composed of a viral capsid protein, but some contain several structural proteins, forming double-layered and triple-layered VLPs. As a representative non-enveloped VLPs, human papillomavirus (HPV) VLPs are well-known as VLP vaccines commercially available. Enveloped VLPs are generally composed of structural proteins and lipid bilayers called an envelope. Therefore, preparing enveloped VLPs in various expression systems is more challenging than non-enveloped VLPs. However, the advantage of enveloped VLPs is the capability to display transmembrane proteins as viral envelope proteins.

Enveloped viruses have viral envelope proteins that bind to host cells' receptors. Some transmembrane proteins of host cells are also displayed on the envelope of viruses [4,5]. As well, recombinant transmembrane proteins can be displayed on the surface of enveloped VLPs by the coexpression of a structural protein of viruses [6,7]. Antigen-displayed enveloped VLPs can produce antigen-specific antibodies and specific cell targeting for the drug delivery system.

Insect cells have already been utilized for the commercial production of HPV-LPs. In addition to insect cells, silkworms have been used for biofactory to produce many

58 recombinant proteins and are a promising host for recombinant protein production [8,9].
59 The enveloped or non-enveloped VLPs have been produced in silkworms [10], e.g.,
60 porcine circovirus type 2 VLPs and bovine papillomavirus type 6 VLPs [11,12].
61 Our previous study produced Rous sarcoma virus-like particles (RSV-LPs) in
62 silkworm larvae by expressing RSV Gag protein lacking PR domain [13,14]. This study
63 tried to generate RSV-LPs displaying two kinds of hemagglutinins (HAs) from the
64 influenza A viruses (VLP/BivHA) in silkworm larvae by coexpressing RSV Gag protein
65 and HAs. Additionally, recombinant proteins were displayed on the surface of RSV-LPs
66 using the transmembrane domain (BmGP64TM) of glycoprotein 64 (BmGP64) from
67 *Bombyx mori* nucleopolyhedrovirus (BmNPV) [15]. The VLP/BivHA's properties were
68 examined using hemagglutination assay against specific receptors and immuno-TEM.
69
70 **2. Materials and methods**
71
72 *2.1. Construction of recombinant BmNPV bacmids*
73 A gene encoding HA of influenza A/H1N1 (A/New Caledonia/20/99) (H1) was
74 purchased from Sino Biological (Beijing, China). Full-length of a gene encoding H1 was
75 amplified by PCR using a primer set (HA-NC-F and -R) as described in Table 1. An
76 amplified DNA fragment was inserted into pFastBac 1 (Thermo Fisher Scientific K. K.,
77 Tokyo, Japan). The resulting plasmid (pFastBac1/H1 in Fig. 1) was transformed into
78 *Escherichia coli* BmDH10bac, and the recombinant BmNPV bacmid containing the gene
79 encoding H1 was extracted from the white colony [16], designating BmNPV/H1 bacmid
80 (Fig. 1).
81 To express HA of influenza A H5N1 (A/Vietnam/1194/2004) (H5), a bx-HA-His

fragment in pFastBac 1 was used [17]. The bx-HA-His fragment has the signal peptide sequence of bombyxin from *B. mori* at the 5'-end and the sequence encoding GS linker (GGGSGGGS) and 6 × His tag at the 3'-end. The bx-HA fragment was amplified by PCR using the primer set (HA-VT-F and -R) described in Table 1, and the amplified DNA fragment was inserted into pFB/GP64 TM [18], leading to the construction of H5 fused with BmGP64TM of BmGP64. The resulting plasmid (pFastBac1/H5 in Fig. 1) was transformed into *E. coli* BmDH10Bac. The recombinant BmNPV bacmid containing the fusion protein gene was extracted from the white colony, designating BmNPV/H5 bacmid (Fig. 1).

Recombinant BmNPV containing a gene encoding Gag protein of Rous sarcoma virus (BmNPV/gag-577 bacmid) was constructed previously [14].

2.2. Expression of recombinant proteins in silkworm larvae

Using procedures previously described [16], recombinant BmNPV bacmid DNA was injected into silkworm larvae that become infected, and from which recombinant BmNPV budded virus (BV) are recovered. Briefly, approximately 10 μg of a recombinant BmNPV bacmid (including the helper plasmid) was mixed with 1/10 volume of DMRIE-C (Thermo Fisher Scientific K. K.) and incubated at room temperature for 30 min. The mixture was injected into the 5^{th} instar of silkworm larvae, and these larvae were raised on an artificial diet, Silkmate S2 (Nosan, Yokohama, Japan), for 6–7 d. One hundred-fold diluted hemolymph was injected into the 5^{th} instars of silkworm larvae. After 3–4 d, hemolymph and fat body were collected. After the recombinant protein expression was confirmed by western blot, the hemolymph was collected and used as a stock of recombinant BmNPV. Titers of recombinant BmNPVs in hemolymph were determined

according to the protocol previously described [19].

To coexpress two or three recombinant proteins in silkworm larvae, each recombinant BmNPV was simultaneously injected into 5^{th} instars of silkworm larvae at 1:1 or 1:1:1, respectively (Fig. 1). After 3–4 d, hemolymph and fat body were collected for assay.

2.3. SDS-PAGE and western blot

The collected fat body was suspended with phosphate-buffered saline (PBS, pH 7.4) and disrupted by sonication. Its homogenate and hemolymph were centrifuged at 7,000 × g to remove insoluble materials and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% acrylamide gel at 70 V. Proteins were blotted onto the polyvinylidene difluoride (PVDF) membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, California, USA) at 15 V for 60 min. The membrane was soaked in blocking buffer containing 5% (w/v) skim milk in Tris-buffered saline (TBS) containing 0.1% (w/v) Tween 20 (TBST) for 1 h. A 1,000 fold-diluted mouse anti-H1N1 IgG monoclonal antibody (Abcam K. K., Tokyo, Japan) and 10,000 fold-diluted anti-mouse IgG(H+L)-horseradish peroxidase (HRP) (Medical and Biological Laboratory, Nagoya, Japan) were used to detect H1 as the primary and secondary antibodies, respectively. To detect H5, 1,000 fold diluted rabbit anti-H5N1 IgG polyclonal antibody (Sino Biological) was used as the primary antibody for HA, followed by 10,000 fold-diluted anti-rabbit IgG (Medical and Biological Laboratory), a secondary antibody. RSV Gag protein was detected by 2,000 fold-diluted serum from mice immunized by the nucleocapsid domain of gag-577 and 10,000 fold-diluted anti-mouse IgG (Medical and Biological Laboratory) sequentially as the primary and secondary

antibody, respectively. A Chemiluminescence kit (Millipore Sigma, California, USA) was used to visualize these specific protein bands and detected using a VersaDoc, Fluor-S/MAX multi-imager (Bio-Rad).

2.4. Purification of VLPs using fetuin agarose chromatography

Hemolymph was diluted 10-fold with TBS and filtered using a 0.80 μm size MF-Millipore membrane filter (Merck, New Jersey, USA). The filtrate was loaded into the 1 mL of fetuin agarose column (Sigma-Aldrich, Missouri, USA). Flowthrough samples were reloaded to the column and repeated 10 times. The gels were subsequently washed with washing buffer (10 mM $CaCl_2$, 33 mM Tris-HCl, and 50 mM NaCl, pH 8.0). VLPs were eluted with 3 mL of elution buffer (10 mM EDTA, pH 8.0). Proteins in the 500 μL of elution fraction were analyzed by western blot. To measure the protein concentration, the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, USA) was used.

2.5. TEM analysis

Fifty μL of purified VLPs were spotted on the carbon grid, dried at room temperature, then stained with 2% phosphotungstic acid as negative staining. After the negative staining, the grid was washed with PBS (pH 7.0) and air-dried at room temperature for a few seconds. Subsequently, the grid was loaded to the transmission electron microscopy (TEM) (JEM-1400Plus, JEOL, Tokyo, Japan).

For immuno-TEM, 50 μL of purified VLPs was dropped on the carbon grid, followed by washing with TBS and drying the carbon grid at room temperature for 12 h. Afterward, the grid was spotted on 1% BSA as a blocking buffer. The grid was soaked with 10 fold-diluted mouse IgG anti-H1 antibody (Abcam K. K.) and rabbit IgG anti-H5N1 polyclonal

antibody (Sino Biological) for 2 h and washed with 1% BSA 3 times. To detect H1 and H5, the grid was soaked with 20-fold-diluted goat anti-rabbit IgG-conjugated 5 nm gold nanoparticles (BBI Solutions, California, USA) and goat anti-mouse IgG-conjugated 15 nm gold nanoparticles (BBI, California, USA), respectively, for 2 h. The grid was washed with 1% BSA, and the negative staining using 2% phosphotungstic acid was conducted. Subsequently, the display of H1 and H5 on the surface of each VLP was observed utilizing the TEM (JEM-1400Plus, JEOL).

Dynamic light scattering (DLS) analysis was performed using a Malvern Zetasizer nanoseries Nano-ZS90 system (Malvern Inst. Ltd., Malvern, UK) to measure the size of purified VLPs.

2.6. Hemagglutination assay

Red blood cells (RBCs) of chicken, sheep, and rabbit (Japan SLC, Hamamatsu, Japan) were prepared from the blood of these animals. A 10 mL of blood was centrifuged to collect RBCs. After collecting RBCs by centrifugation, the RBCs were washed with PBS 5 times and suspended with PBS. Then, an equal volume of 5% trypsin solution was added to the suspension of the RBCs, and the suspension was incubated with gently stirring for 1 h. VLPs (0.1 mg/μL) were serially diluted with PBS in a 96-well plate with a volume of 50 μL, and 50 μL of RBC solution was added to each well. The 96 well plates were incubated at room temperature for 3 h. Gag protein partially purified by sucrose density gradient centrifugation was used as a negative control.

3. Results

178

179 *3.1. Expression of H1, H5, and Gag protein in silkworm larvae*

180 A full-length H1 with its transmembrane domain was expressed in the hemolymph
181 and fat body as HA0 (65 kDa in Fig. 2A). BmGP64TM-fused H5 was expressed in
182 hemolymph and fat body and detected HA0, HA1, and HA2 at 65 kDa, 45 kDa, and 28
183 kDa, respectively (Fig. 2B). HA0 is a full-length of HA but might be cleaved into HA1
184 and HA2 due to proteolytic activity [20]. When we expressed H1 and H5 as a secretory
185 proteins in the hemolymph of silkworm larvae, the cleaved HA1 and HA2 were observed.
186 Gag protein of molecular weight of 61 kDa was detected in hemolymph and the fat body
187 in silkworm larvae (Fig. 2C), which is the same molecular weight as has been previously
188 reported [14].

189

190 *3.2. Purification of RSV-LPs displaying HAs*

191 Production of RSV-LPs displaying H1 or H5 was performed by the coexpression of
192 RSV Gag protein and H1 or H5 (Fig. 1). RSV-LPs composed of Gag protein are secreted
193 into hemolymph in silkworm larvae as a VLP [14]. In addition, HA binds to α-2,3 and/or
194 α-2,6-sialylated glycan, which makes the HAs purify by fetuin agarose chromatography
195 [17]. Therefore, RSV-LPs displaying HAs were purified using fetuin agarose column
196 chromatography from the larval silkworm hemolymph coexpressing RSV Gag protein
197 and H1 (VLP/H1) or H5 (VLP/H5) (Fig. 3A). In elution fraction 1 of H1 (VLP/H1), HA0,
198 HA1, and HA2 were observed. HA0, HA1, and HA2 of H5 (VLP/H5) were observed in
199 elution fractions of E1–E3. RSV Gag protein of VLP/H1 or /H5 was detected by western
200 blot (lower panel of Fig. 3A). These results indicate that the coexpression of RSV Gag
201 protein and each HA in silkworm larvae led to forming VLP/H1 and VLP/H5 in the

hemolymph. The purified amount of VLP/H1 and /H5 was 8.78 and 9.32 μg from one silkworm larva, respectively. TEM images of VLP/H1 and /H5 were smooth, round spherical shape (Fig. 3B), and the diameter of VLP/H1 or /H5 was around 70–80 nm (Fig. 3C). Both TEM and DLS data show approximately similar diameter sizes.

The RSV-LPs displaying both HAs were prepared by coexpression of RSV Gag protein, H1, and H5 (Fig. 1). RSV-LPs displaying both HA (VLP/BivHA) were purified from the hemolymph of silkworm larvae coexpressing RSV Gag protein and both HAs using fetuin agarose column chromatography. The purified amount of VLP/BivHA was 8.22 μg from one silkworm larva, similar to that of VLP/H1 or /H5. HA0 of VLP/H1, HA0–HA2 of VLP/H5, and Gag protein were detected in the elution fractions (Fig. 4A). TEM analysis revealed this coexpression of RSV Gag protein, H1, and H5 formed RSV-LPs (Fig. 4B). The diameter of VLP/BivHA was around 70–80 nm (Fig. 4C). Both TEM and DLS data show approximately similar diameter sizes.

Additionally, by the immuno-TEM analysis, different sizes of gold nanoparticles were attached to the surface of VLP/BivHA (Fig. 5). Dotted arrows indicate 5 nm of gold nanoparticles, suggesting H1 presence: solid arrows 15 nm of gold nanoparticles, pointing to H5 presence. These images suggest that H1 and H5 were displayed on the surface of REV-LPs with HA and GP64 transmembrane proteins, respectively. These results indicate that VLP/BivHA was expressed in the hemolymph of silkworm larvae and purified from the hemolymph using fetuin agarose column chromatography.

3.3. Hemagglutination assay of VLPs/H1, /H5, and /BivHA

To confirm the display of both HAs on the RSV-LPs, the binding assay was performed using RBCs of chicken, sheep, and rabbit. RBCs of chicken have both α2,3-sialyl- and

α2,6-sialylglycoproteins. On the other hand, RBCs of sheep and rabbit have either α2,3-sialylglycoprotein or α2,6-sialylglycoproteins. H1 is an influenza A/H1N1 and binds to α2,6-sialylglycans [21]. H5 is an influenza A/H5N1 and binds to α2,3-sialylglycans [17]. The VLPs/H1 were able to agglutinate the RBCs of chicken and rabbit, but not sheep (Fig. 6A). On the contrary, VLPs/H5 showed hemagglutination activity against the RBCs of chicken and sheep, but not rabbit (Fig. 6B). These results indicate that VLPs/H1 and VLPs/H5 prepared in silkworm larvae were functional. Additionally, the VLPs/BivHA were able to agglutinate the RBCs of all three animals (Fig.6C). This result indicates that VLPs/BivHA prepared in silkworm larvae were also functional. RSV-LPs partially purified from hemolymph did not have any capacity to hemagglutinate RBCs of all three animals (Fig. 6D).

4. Discussion

In this study, VLPs/BivHA, which have two HAs on the surface of RSV-LPs, were prepared in silkworm larvae by the coexpression of RSV Gag protein, H1, and H5. H5 was fused to the transmembrane domain of BmGP64, which is an envelope protein in BmNPV. In our previous study, the transmembrane domain of BmGP64 allows recombinant proteins to be displayed on the surface of RSV-LPs [15]. This study showed the display of H5 on the RSV-LPs using this transmembrane domain. H1 has its own transmembrane domain, and H1 was also displayed on the RSV-LPs via its transmembrane domain. This result indicates that the transmembrane domain of HA can be used to display recombinant proteins on the RSV-LPs as well as that of BmGP64. Then, it supposes that the transmembrane domains of envelope proteins from enveloped viruses may allow the display of recombinant proteins on the surface of eVLPs.

Hemagglutination test reveals that VLPs/H5 hemagglutinate the RBCs of sheep up to 8-fold dilution, but VLPs/H1 those of rabbit up to 16-fold dilution. This means H5 may be displayed two times higher on the surface of VLP than H1. From Immuno-TEM analysis, the number of H5 was higher than that of H1 (Fig. 5). It is challenging to display H1 and H5 on the surface of VLPs uniformly by this coexpression system.

Even though some techniques have been used [22–24], the purification of eVLPs is more laborious than that of non-enveloped VLPs. Significantly, the hemolymph of silkworm larvae has a high concentration of intrinsic proteins. In this study, RSV-LPs/BivHA were purified from hemolymph by fetuin agarose column chromatography via the binding of HAs to sialylated *N*-glycan of fetuin. Even though the western blotting of the purified sample shows the target band of each protein, it is not convincing enough to indicate that our VLPs have displayed H1 or H5 on VLPs/H1, H5, and /BivHA.

Hemagglutination assay and immuno-TEM confirmed that the purified VLPs/H1, H5, and /BivHA were successfully displayed H1 and H5. Hemagglutinin has a specific binding against each specific binding receptor as it was supposed to be. For VLPs/BivHA, the hemagglutination against both receptors indicates that they have both H1 and H5. The display of two HAs on the surface of VLPs/BivHA was also observed by immuno-TEM.

In conclusion, the VLPs/BivHA displaying dual HAs, H1 and H5, were produced in silkworm and showed similar hemagglutination against the sialylglycopeptides α-2,3 or α2,6 receptors. The VLPs/BivHA will be forwarded to animal experiments to demonstrate immunogenesis targeting for the vaccine platform.

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364

365

366 Table 1. Primers

	Sequence (5’-3')
HA-NC-F	CGGGATCCATGAAGGCTATCCTGGTGGTGCTGCTCTACACCTTTGCCACA GCCAATGCTGACACCCTGGACTACAAGGATGACG
HA-NC-F	GGGGTACCTTAGATACAAATCCTACATTGGAGGGA
HA-VT-F	CGGGGATCCATGAAGATACTCCTTGCTATTG
HA-VT-R	CGGGGTACCAGAACTCGCCACTGTTGAATAAATTG

367

Figure legends

Fig. 1. Schematic representation of VLP generation by coexpression using silkworm. A gene encoding HA signal sequence, a FLAG tag, and HA of influenza A/H1N1 (A/New Caledonia/20/99) (H1) were inserted into pFastBac 1. The resulting plasmid (pFastBac1/H1) was transformed into *Escherichia coli* BmDH10bac, and the recombinant BmNPV/H1 bacmid was obtained. To express HA of influenza A H5N1 (A/Vietnam/1194/2004) (H5), a bombyxin signal sequence (bx)-6×HA-His-H5-fused BmGP64TM was inserted into pFastBac 1 and resulting pFastBac1/H5 was transformed into *E. coli* BmDH10bac, and the recombinant BmNPV/H5 bacmid was obtained. To coexpress two or three recombinant proteins in silkworm larvae, each recombinant BmNPV was simultaneously injected into 5^{th} instars of silkworm larvae at 1:1 or 1:1:1, respectively.

Fig. 2. Expression of H1 (A), H5-TM (B), and RSV Gag (C) proteins in silkworm larvae. Hemolymph and extraction of the fat body were prepared according to the "Materials and methods," and expressed recombinant proteins were detected by western blot. M, H, FB, and PC indicate molecular marker, hemolymph, fat body extract, and the positive control of RSV Gag protein, respectively.

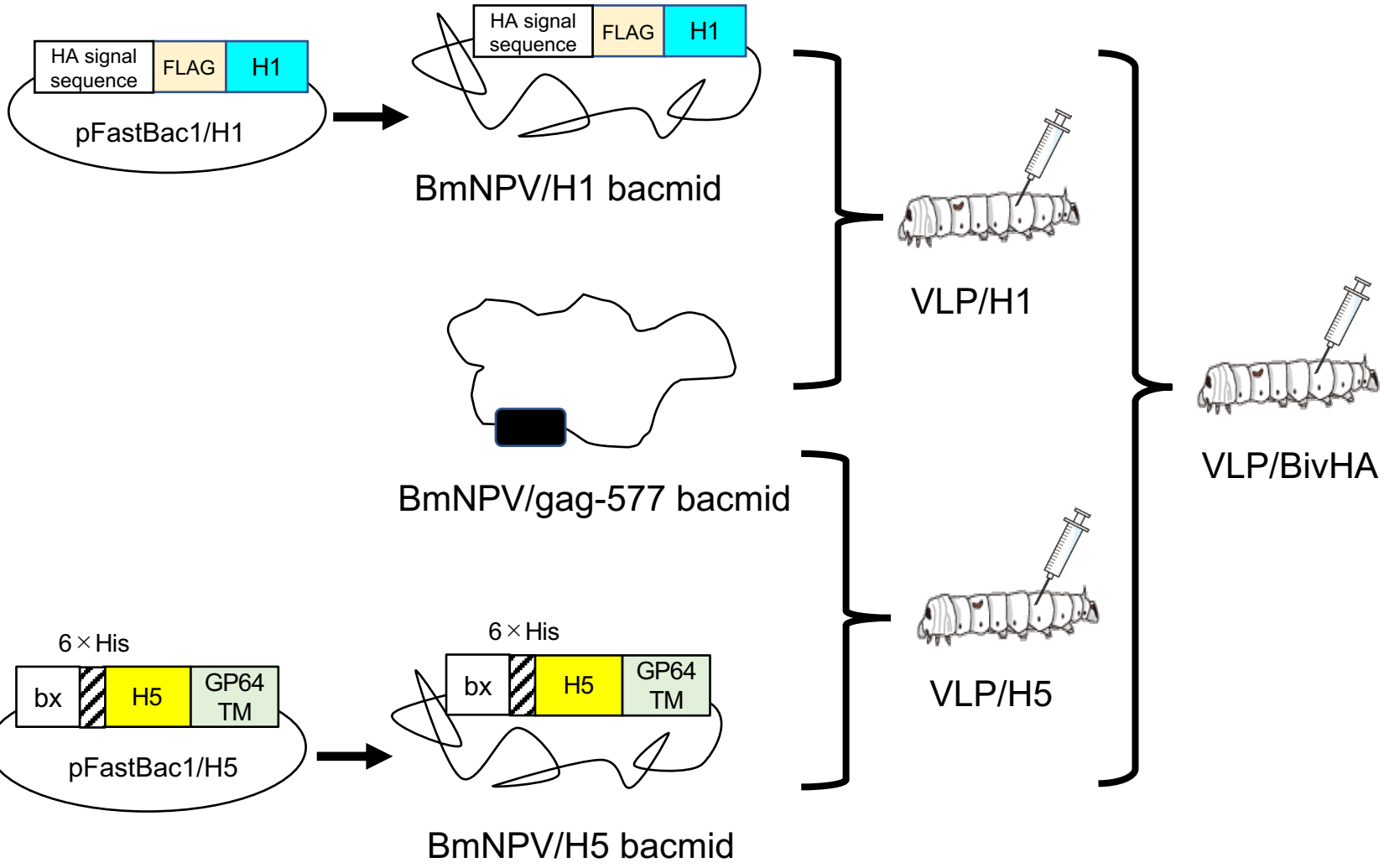
Fig. 3. Purification of VLP/H1 and VLP/H5 from hemolymph using fetuin agarose column chromatography (A). VLPs were purified from hemolymph using fetuin agarose column chromatography according to the "Material and methods," and eluted proteins were analyzed by western blot using each anti-HA antibody and anti-Gag serum. H indicates hemolymph; 1, 2, and 3, elution fraction number. TEM images (B) and DLS analysis (C) of purified VLP/H1 and VLP/H5. Eluted each RSV-LP was analyzed by TEM

391 and DLS according to the "Materials and methods."

392 **Fig. 4.** Purification of VLP/BivHA (A) Purification of VLP/BivHA from hemolymph
393 using fetuin agarose column chromatography. VLP/BivHA was purified from hemolymph
394 using fetuin agarose column chromatography according to the "Material and methods,"
395 and eluted proteins were analyzed by western blot using each anti-HA antibody and anti-
396 Gag serum. Hem indicates hemolymph. TEM image (B) and DLS analysis (C) of purified
397 VLP/BivHA. Eluted VLP was analyzed by TEM and DLS according to the "Materials
398 and methods".

399 **Fig. 5.** Immuno-TEM image of VLP/BivHA. Eluted VLP/BivHA was analyzed by TEM
400 according to the "Materials and methods." To detect H1 and H5, the TEM-grid was soaked
401 with 20-fold-diluted goat anti-rabbit IgG-conjugated 5 nm gold nanoparticles for H1
402 (dotted arrows) and goat anti-mouse IgG-conjugated 15 nm gold nanoparticles for H5
403 (solid arrows), respectively.

404 **Fig. 6.** Hemagglutination assay of VLP/H1 (A), VLP/H5 (B), VLP/BivHA (C), and RSV-
405 LPs (D). RBCs were prepared from chicken, sheep, and rabbit blood, and
406 hemagglutination assay was conducted according to the "Materials and methods." RSV-
407 LPs partially purified by sucrose density gradient centrifugation were used as a negative
408 control. Numbers in the left denote serial dilution of VLPs with PBS.


HA signal sequence
FLAG
H1
pFastBac1/H1
HA signal sequence
FLAG
H1
BmNPV/H1 bacmid
VLP/H1
BmNPV/gag-577 bacmid
VLP/BivHA
6×His
bx
H5
GP64 TM
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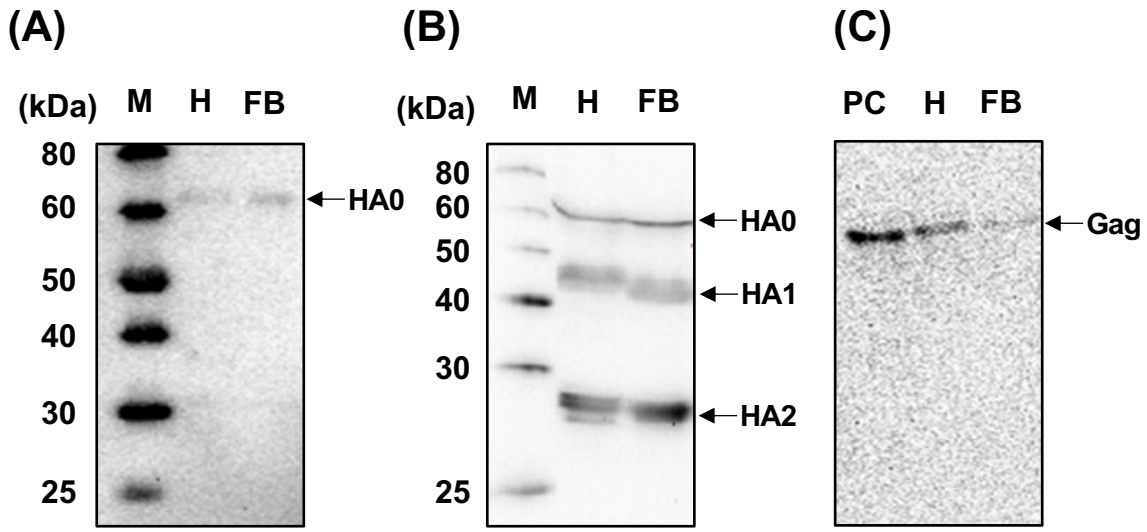
6×His
bx
H5
GP64 TM
BmNPV/H5 bacmid
VLP/H5


(A)
(kDa)
M
H
FB
80
60
50
40
30
25
HA0
(B)
(kDa)
M
H
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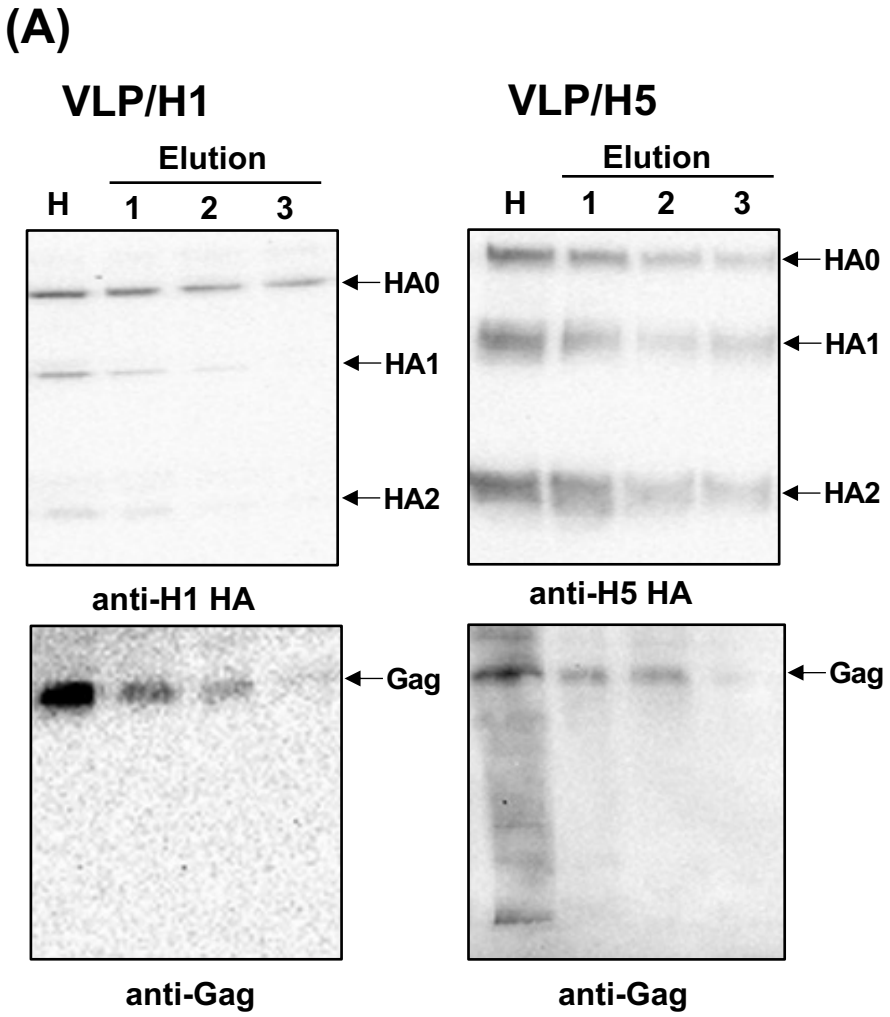
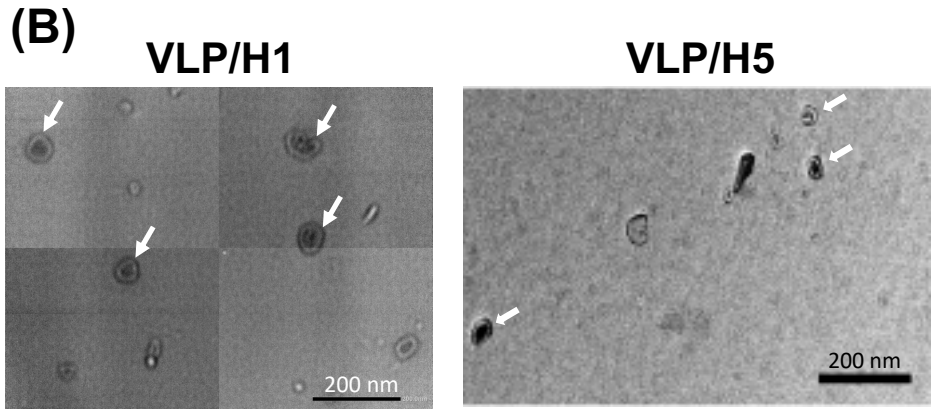
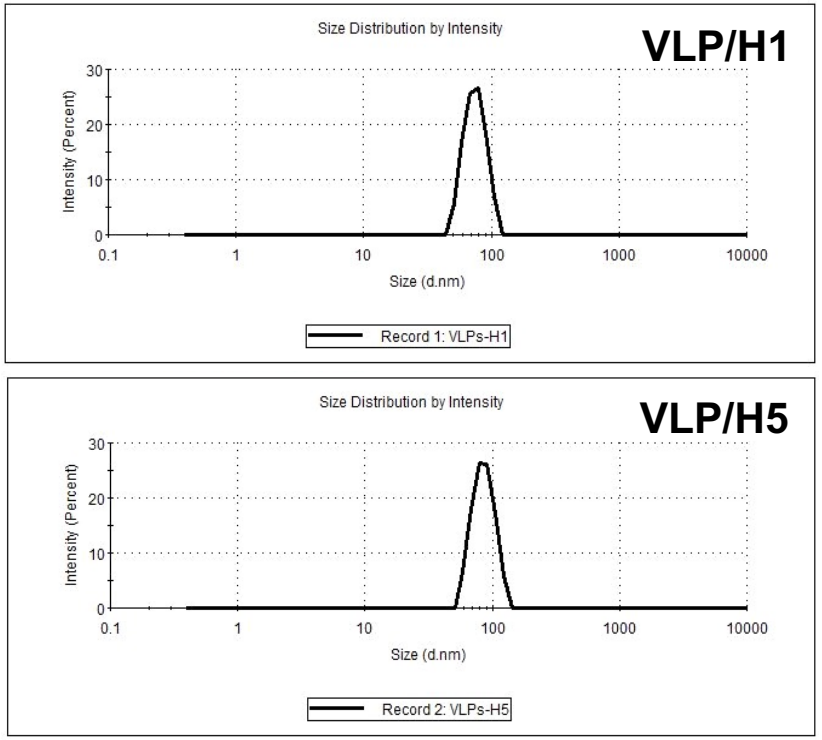
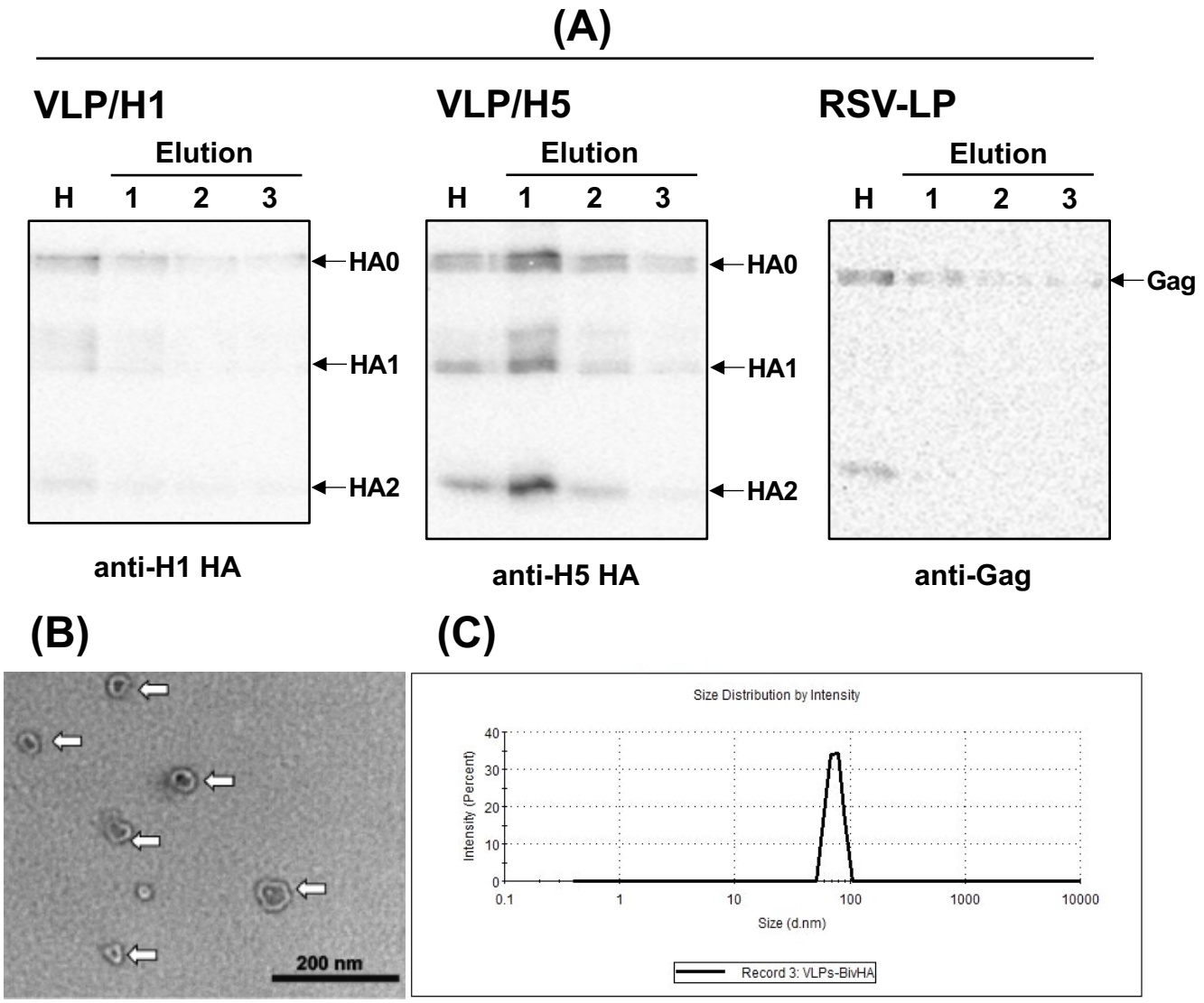
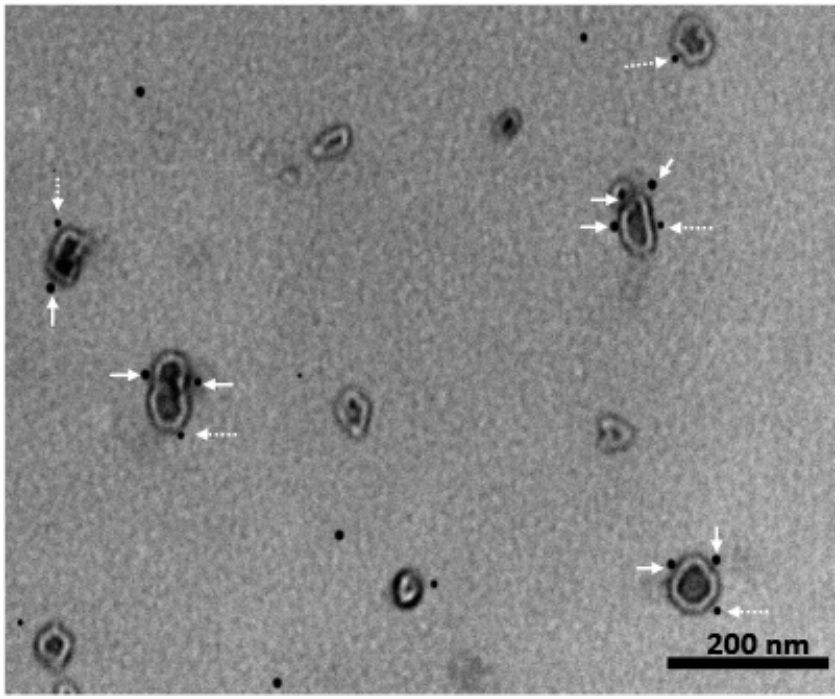
HA0
HA1
HA2
(C)
PC
H
FB
Gag

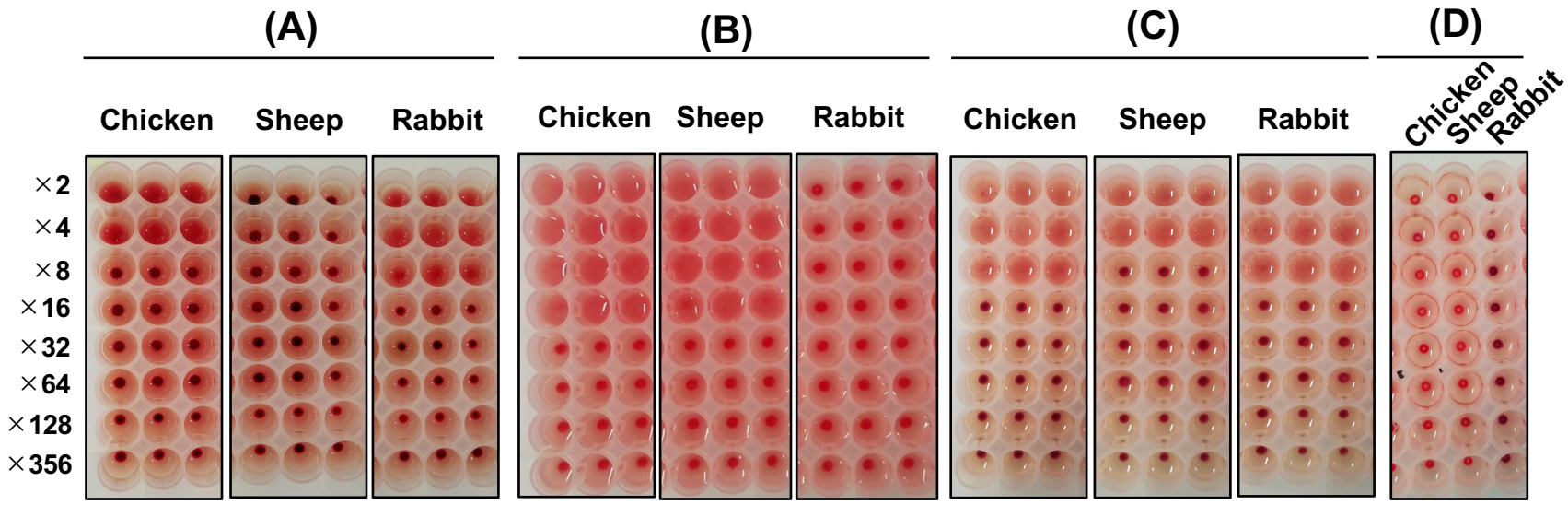

(A)
VLP/H1
VLP/H5
Elution
H
1
2
3
HA0
HA1
HA2
anti-H1 HA
anti-H5 HA
Gag
anti-Gag


(B)
VLP/H1
VLP/H5
200 nm
200 nm


(C)
Size Distribution by Intensity
VLP/H1
Intensity (Percent)
Size (d.nm)
Record 1: VLPs-H1
Size Distribution by Intensity
VLP/H5
Intensity (Percent)
Size (d.nm)
Record 2: VLPs-H5


(A)
VLP/H1
VLP/H5
RSV-LP
Elution
H
1
2
3
HA0
HA1
HA2
Gag
anti-H1 HA
anti-H5 HA
anti-Gag
(B)
200 nm
(C)
Size Distribution by Intensity
Intensity (Percent)
Size (d.nm)
Record 3: VLPs-BivHA


200 nm


(A)
(B)
(C)
(D)
Chicken
Sheep
Rabbit
Chicken
Sheep
Rabbit
Chicken
Sheep
Rabbit
Chicken
Sheep
Rabbit
×2
×4
×8
×16
×32
×64
×128
×356