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Surface Labeling of Enveloped Viruses Assisted by Host Cells

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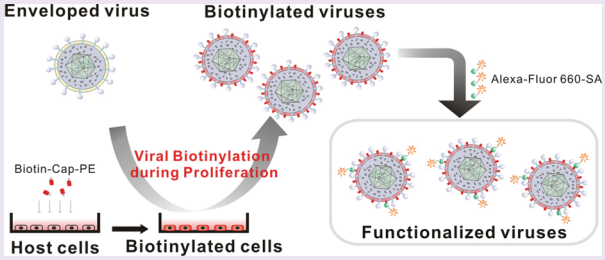
Supporting Information

ABSTRACT: Labeling of virus opens new pathways for the understanding of viruses themselves and facilitates the utilization of viruses in modern biology, medicine, and materials. Based on the characteristic that viruses hijack their host cellular machineries to survive and reproduce themselves, a host-cell-assisted strategy is proposed to label enveloped viruses. By simply feeding Vero cells with commercial 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl) (sodium salt) (Biotin-Cap-PE), we obtained biotinylated Vero cells whose membrane systems were modified with biotin. Subsequently, pseudorabies viruses (PrV) were cultivated in the biotinylated Vero cells, and the PrV progenies were spontaneously labeled with Biotin-Cap-PE during viral natural assembly process. Since the viral natural assembly process was employed for the labeling, potential threats of genetic engineering and difficulties in keeping viral natural bioactivity were avoided. Importantly, this labeling strategy for enveloped virus greatly reduces the technical complexity and allows researchers from different backgrounds to apply it for their specified demands.



As the most abundant type of organisms, viruses are found in almost every ecosystem on the earth. Nowadays, viruses not only are infectious agents but also are emerging as platforms at the nanoscale for diverse applications in biomedicine,[1−3] biomaterials,[4,5] and nanotechnology.[6,7] By labeling, new functions can be imparted to viruses according to different demands.[8,9] Generally, there are three approaches for the labeling of viruses: genetical engineering,[10−12] chemical coupling,[13,14] and physicochemical association.[15,16] However, the complex processes, technical barriers, and even the inactivation in labeling or subsequent purification limit viral application, especially to enveloped viruses that are more vulnerable to hydrodynamic stress and heat than nonenveloped viruses. Recently, some inspiring improvements in the labeling of enveloped viruses were reported. Mukherjee *et al.* achieved viral labeling through dynamic exchange with functional lipids.[17] Chen *et al.* and Cheng *et al.* utilized the budding process of enveloped viruses to achieve viral modification, respectively.[18,19] Yet it is still needed to find a simpler and faster labeling method for enveloped viruses without damaging viral native activities.

Herein, a host-cell-assisted labeling strategy for enveloped viruses is proposed. Based on the characteristic that viruses hijack their host cellular machineries to survive and reproduce themselves, the labeled viruses' activity was preserved to the largest extent. As shown in Figure 1, our strategy is to prefeed host cells with biotin functionalized phosphatidylethanolamine (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl) (sodium salt), Biotin-Cap-PE, Supplementary Figure 1), so that Biotin-Cap-PE molecules can be taken up and distributed in the whole membrane systems including the viral budding sites through cellular uptake and membrane traffic. Phosphatidylethanolamine, a major component of cells, was chosen as a membrane anchoring unit here because it is a structural component in both cells and virions,[20] unlike sphingolipids and cholesterol that were reported to play important roles in viral budding and entry.[21] Pseudorabies virus (PrV), a dsDNA enveloped virus, was employed as a model enveloped virus to be biotinylated in their host cells (kidney epithelial cells of the African Green Monkey, Vero cells). When enveloped PrV virions proliferate in biotinylated Vero cells, they could naturally wear biotinylated envelopes during their budding.[22−24]

Fluorescence microscopic imaging, flow cytometry assay, and confocal fluorescence microscopic imaging were used to evaluate the biotinylation effect of the cells after labeling with streptavidin-Cy3 conjugates (Cy3-SA). In Figure 2a, fluorescence was observed in all of the biotinylated Vero cells incubated with Cy3-SA but not in normal Vero cells. Figure 2b

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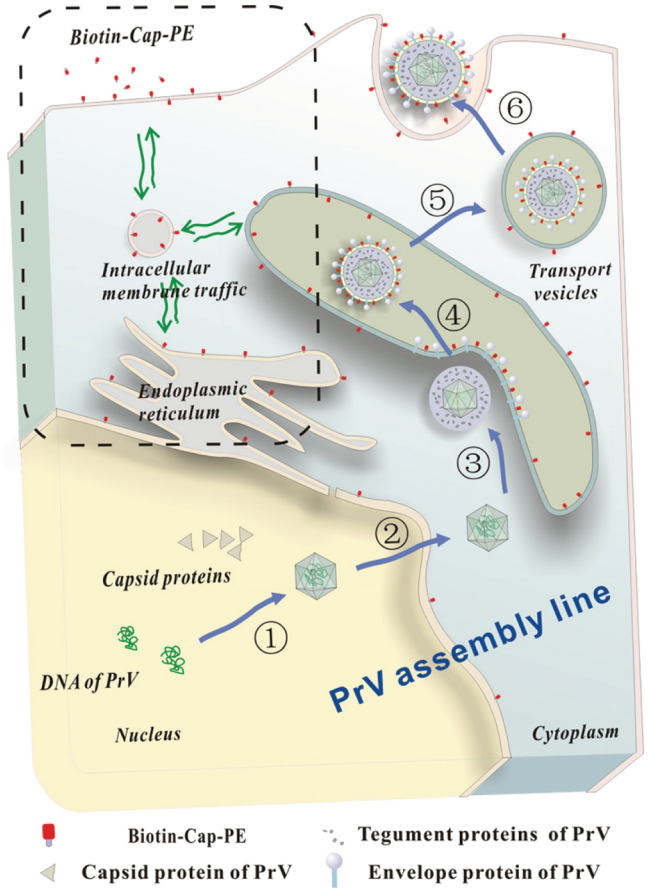

Figure 1. Schematic illustration of PrV assembly with Biotin-Cap-PE in the host cell. After the replication of viral genome in the nucleus of the host cell, the progeny genome of PrV packages into a preformed capsid to form a nucleocapsid (1). After crossing the nuclear membrane (2), the PrV nucleocapsid packs with viral tegument proteins in the cytoplasm (3) and buds on the cytoplasmic face of a specialized compartment derived from the trans-Golgi network containing viral envelope proteins and Biotin-Cap-PE (4). Biotin-Cap-PE molecules are assembled in the envelope of the mature PrV at the budding site. Then the mature biotinylated virion is transported within a cellular vesicle (5). On the arrival at the cell surface, the vesicle fuses with cell membranes and finally releases a mature, biotinylated PrV virion from the cell (6).[25,32] In the dotted line box, the pathways of the internalization and distribution of Biotin-Cap-PE through cellular uptake and membrane traffic is illustrated according to previous reports.[22−24]

shows flow cytometry assay results of cells with different incubation times, which indicated that the concentration of cellular Biotin-Cap-PE increased with the incubation time and finally became steady after 3 days' incubation. These results suggested that Vero cells could be biotinylated by simply cultivating them in Biotin-Cap-PE containing medium for several days. Further observations with confocal fluorescence microscope indicated that Cy3-SA binding with Biotin-Cap-PE not only stayed at the cell membranes but also internalized into the cells (Figure 2c). The bustling transport of the Cy3-SA (see Supplementary Movie) also suggested that Biotin-Cap-PE molecules could spontaneously join in the cellular membrane traffic systems. Moreover, MTT assay and continuous passage cultivation showed no obvious toxicity of Biotin-Cap-PE to cell growth and proliferation (Figure 2d). All of the above results suggested that Biotin-Cap-PE could be incorporated into cellular membrane systems without disturbing cell growth and proliferation.

PrV viruses were cultivated in the as-prepared biotinylated Vero cells with similar procedures in common viral culture. After 2 days' cultivation, viral progenies egressed from host cells. The biotinylation efficiency was evaluated by fluorescence microscopic colocalization assay of the virions whose DNA were dyed with SYTO 82 and envelopes were labeled with Alexa-Fluor 660-SA. As can be seen in Figure 3, most fluorescence signals of SYTO 82 colocalizated with that of Alexa-Fluor 660 (Figure 3, second row). The colocalization efficiency of the resulted virions was 89.5 ± 3.2% (mean ± SD). On the contrary, no Alexa-Fluor 660 signal was observed for the normal PrV virions grown in the normal medium without Biotin-Cap-PE (Figure 3, first row). Therefore, most virions egressed from the biotinylated cells had been modified with Biotin-Cap-PE.

PrV's budding site is the cytoplasmic face of a specialized compartment derived from trans-Golgi network in host cells.[25] The mature virions inside biotinylated host cells were extracted, purified, and analyzed with the colocalization assay. In our experiments, the colocalization efficiency of the extracted PrV progenies was 80.4 ± 5.8% (mean ± SD) (Figure 3, third row), much higher than that of the PrV virions by simple incubation with Biotin-Cap-PE (39.9 ± 6.6%, Figure 3, fourth row). These results verified that the biotinylation of PrV mainly finished in Vero cells during virus budding. In the whole process, host cells act as a mild "reactor" assisting the incorporation of Biotin-Cap-PE into the viral envelope, so that biotinylation could be achieved with higher efficiency.

The cell-assisted biotinylated virions were captured by 200 nm SA-PEG-magnetic beads (Supplementary Figure 2a) and subsequently identified by PCR assay for the gG gene of PrV.[26] Control experiments were conducted on normal PrV or biotin blocked SA-PEG-magmatic beads, respectively. After incubating with biotinylated PrV, the SA-PEG-magnetic beads turned cloudy. Then they were further dyed with SYTO 82 and observed with a fluorescence microscope. In Figure 4left-B, green fluorescent PrV virions agglutinated with the SA-PEG-magnetic beads to form large complexes because of the cross-linking (Figure 4left-B and Supplementary Figure 2b). Meanwhile, the beads were well dispersed, and no fluorescence was observed in the two controls (Figure 4left-A and left-C). After PCR, with electrophoresis, the expected PCR products (294 bp) were detected in the magnetic precipitates of SA-PEG-magnetic beads captured biotinylated virions and the supernatant of the two controls (Figure 4right). These results confirmed that the PrV progenies cultivated with Biotin-Cap-PE modified cells were successfully labeled with biotin and could be captured with SA-PEG-magnetic beads. The absence of the expected PCR fragments for the magnetic supernatant can be attributed to the fact that PCR in our experimental condition is not sensitive enough to detect trace nonbound PrV in the magnetic supernatant, which was consistent with the high biotinylation efficiency (Supplementary Figure 3).

The activity of cell-assisted biotinylated viruses was evaluated by one-step growth kinetics.[27] As shown in Figure 5, no obvious difference was observed compared with that of normal PrV. Both the biotin labeled and the normal PrV could reach 10^8 pfu mL^{-1} at 24 h after infection (multiplicity of infection (MOI) = 3). Moreover, the PrV in continuous cultivation with biotinylated Vero cells were also monitored. Results showed that even after a continuous cultivation for over five generations

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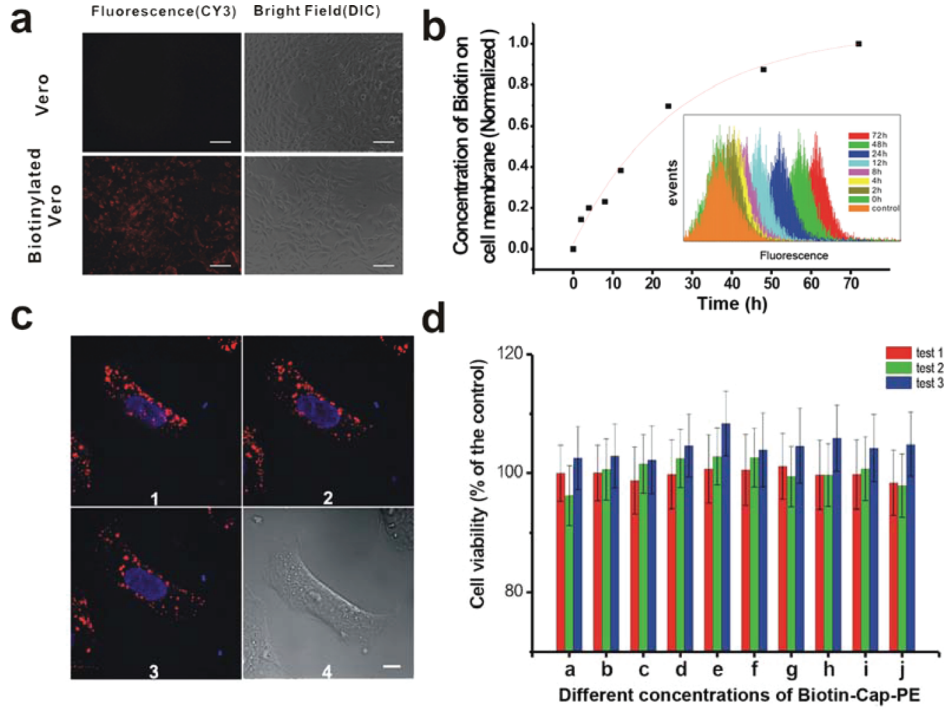

Figure 2. Characterization of Biotin-Cap-PE modified host cells. (a) Fluorescence microscopic imaging of the biotinylated cells labeled with Cy3-SA (Red fluorescence). Scale bar, 100 μm. (b) Flow cytometry assay for monitoring the dynamic process of cellular biotinylation. Each small black square represents the cellular mean fluorescence intensity counted over 10,000 cells at different Biotin-Cap-PE incubation times. The inset in panel b is the raw data that display each signal (Cy3 fluorescence intensity) on the *x*-axis and the number of events (cell count) on the *y*-axis. (c) Confocal imaging of the biotinylated cells targeted by Cy3-SA (Red fluorescence). Different focal planes (images 1, 2, and 3) of biotinyltated Vero cells and the corresponding bright field image (image 4). The step distance between two adjacent focal planes in this figure was 1.092 μm. Scale bar, 10 μm. (d) MTT assay for the toxicity of different Biotin-Cap-PE concentrations. Tests 1, 2, and 3 were three independent experiments. (a–j are different 2-fold serial dilutions of Biotin-Cap-PE from 0.04 mg mL^{-1} to 0.078 μg mL^{-1}).

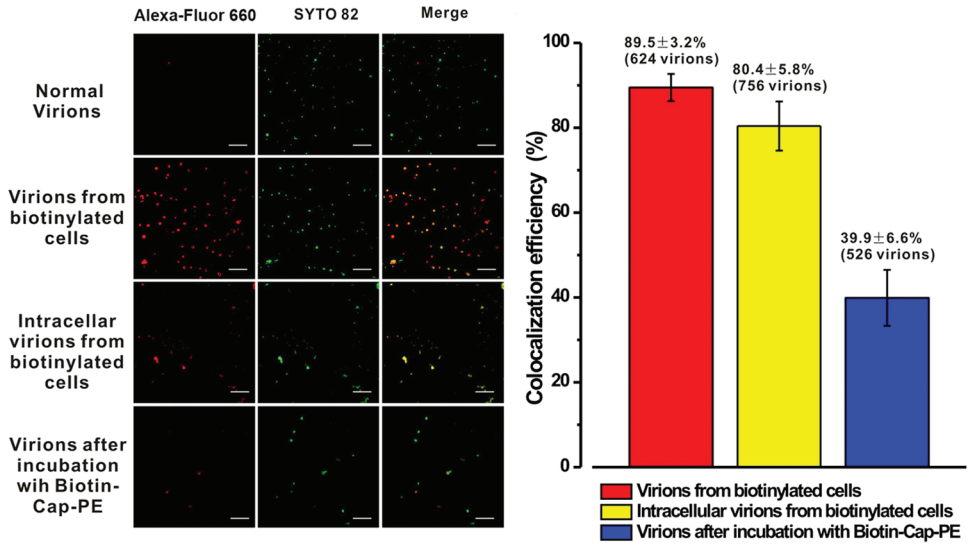

Figure 3. Colocalization assay of biotinylated PrV. (Left) Colocalization of Biotin-Cap-PE inserted in the envelopes and DNA inside the nucleocapsids of normal PrV virions (control, first row), virions from biotinylated cells (second row), intracellular virions from biotinylated cells (third row), and virions after incubation with Biotin-Cap-PE (fourth row). The DNA was stained with SYTO 82 (green) and the Biotin-Cap-PE was labeled with Alexa-Fluor 660-SA (red). Scale bar, 10 μm. (Right) Histogram of the colocalization efficiency of biotinylated PrV.

the biotinylated PrV also kept their infection activity. The biotinylated PrV of each generation could induce the cytopathogenic effects including the rounding of infected cells and the expression of GFP in cytoplasm (Supplementary Figure 4). The titer of the biotinylated PrV after continuous cultivation for five generations reached 10^8 pfu mL^{-1}, which was similar to that of the original generation. Thus it can be concluded that the problem of preserving viral activity was preferably solved by the incorporation of the labeling process into viral assembly. Since the whole viral biotinylation performed spontaneously in the viral replication cycle, operations such as incubation with conjugate chemicals and additional purification before and/or

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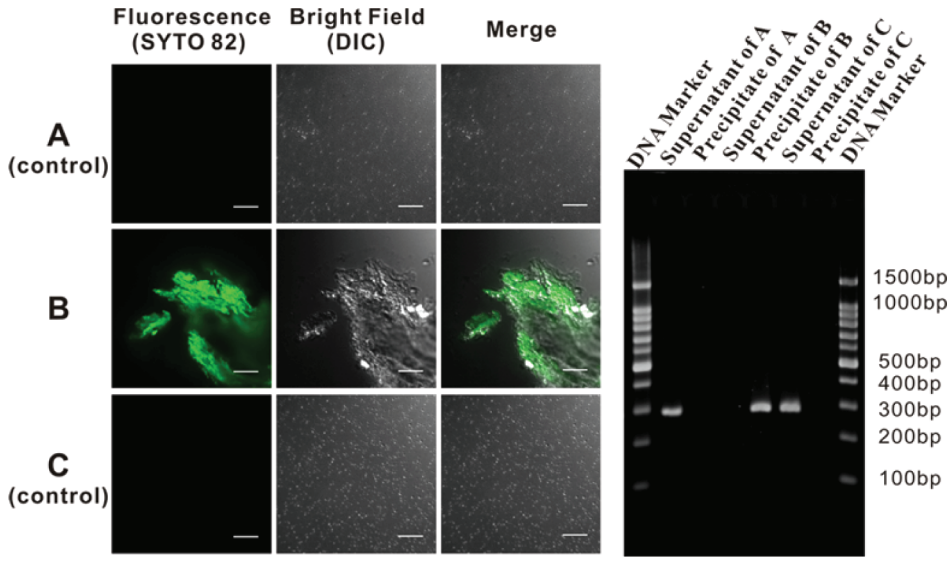

Figure 4. Microscopic images of magnetic precipitates and electrophoretic profiles from subsequent PCR detection. (Left) The biotinylated PrV virions captured with SA-PEG-magnetic beads with (A) or without (B) free biotin blocking or normal virions incubated with SA-PEG-magnetic beads (C). The PrV virions were stained with SYTO 82 (green) and observed under a confocal fluorescence microscope. Scale bar, 10 μm. (Right) PCR detection of PrV in the magnetic precipitates and supernatants from A, B and C. After PCR and electrophoresis, the specific viral target DNA fragment (294 bp) was detected in the magnetic precipitates of B but not in that of A and C. On the contrary, viral target DNA fragments were detected in the supernatant of both A and C but not in the supernatant of B.

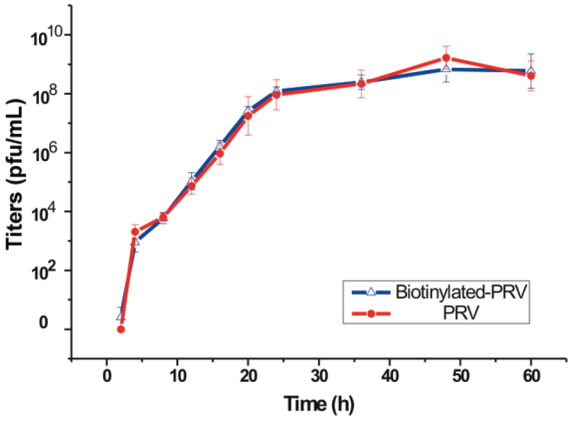

Figure 5. One-step growth curve of PrV and biotinylated PrV on Vero cells. At each time point (0, 2, 4, 8, 12, 16, 20, 24, 36, 48, and 64 h) after inoculation (MOI = 3), both PrV and biotinylated PrV were titrated on common Vero cells for three times. The red dots represent PrV, and the blue triangles represent biotinylated PrV.

after the conjugation can be avoided. Thus viral bioactivity can be kept to the greatest extent, compared with traditional methods.

Different viruses have different budding sites including the endoplasmic reticulum, nucleus membrane, golgi, and other membrane organelles.[28] Because of membrane fluidity, Biotin-Cap-PE may distribute in all of these various viral budding sites. Thus this host-cell-assisted labeling strategy theoretically fits for all kinds of enveloped viruses. Two other important model viruses, baculovirus and poxvirus, were also successfully biotinylated with their biotinylated host cells, respectively. The colocalization efficiency of viral DNA and Biotin-Cap-PE in the viral envelope reached 98.4 ± 0.4% (counted 660 virions) for poxvirus and 87.2 ± 4.6% (counted 639 virions) for baculovirus (Supplementary Figure 5). Unlike PrV, the budding site of baculovirus was the cell membrane,[29] whereas that of poxvirus was somewhere near the microtubule organizing center (MTOC).[30] Our results suggested that this host-cell-assisted strategy is versatile for the labeling of multifarious enveloped viruses with different budding sites.

In summary, a host-cell-assisted labeling strategy for enveloped viruses with high efficiency, good versatility, simple procedures, and low technical barriers is proposed, offering a good example for solving problems with biological systems. Taking advantage of the viral natural assembly process inside host cells, the viruses' activity can be preserved to the largest extent. This strategy may be applied to conveniently modify or label the poorly known enveloped viruses in the face of a new viral lethal outbreak. It can also be combined with other labeling strategies and give researchers more choices. Furthermore, as the role of viruses is transforming from traditional disease-causing agents to potentially vast and beneficial biomaterial resources,[8] the host-cell-assisted labeling strategy reported here opens a clever pathway to artificially functionalize and manipulate enveloped viruses, making enveloped viruses more powerful tools for different demands.

METHODS

Cell Culture and Biotinylation. Normal Vero cells (kidney epithelial cells of the African Green Monkey) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% (w/v) fetal bovine serum (FBS, GIBCO). The biotinylation of Vero cells was achieved by cultivating them in a modified DMEM medium containing 0.02 mg mL^{-1} Biotinyl-Cap-PE (Avanti) and 10% (w/v) FBS. The Sf9 cells were cultivated in supplemented Grace's insect medium with 10% (w/v) FBS, and their biotinylation was achieved by cultivating Sf9 cells in Grace's insect medium containing 0.02 mg mL^{-1} Biotin-Cap-PE and 10% (w/v) FBS.

Virus Culture and Biotinylation. PrV used in this work was a genetically modified virus strain whose genome was inserted with a green fluorescent protein (GFP) gene as the report gene. They were cultivated by almost the same procedure as previous reported.[31] Normal PrV virus was cultivated in normal Vero cells in DMEM supplemented with 2% (w/v) FBS or above-mentioned biotinylated

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Vero cells in DMEM supplemented with 2% (w/v) FBS and 0.02 mg mL^{-1} Biotin-Cap-PE, respectively. Poxvirus was cultivated in biotinylated Vero cells in DMEM supplemented with 2% FBS and 0.02 mg mL^{-1} Biotin-Cap-PE, while baculovirus was cultivated in Sf9 cells in Grace's insect medium containing 0.02 mg mL^{-1} Biotin-Cap-PE and 10% (w/v) FBS.

Colocalization of Viral DNA and Biotin-Cap-PE Inserted in the Viral Envelope. The purified normal or biotinylated virus solution was dropped onto slides (22 mm × 22 mm, Citoglas) cleaned with plasma (PDC-M Chengdu Weike Spectral Devices Techoloies, Inc.). Then the virus was fixed with heat. The DNA of virus was stained by incubation in 0.5 mmol L^{-1} SYTO82 (Molecular Probes) solution. After being blocked with 2% (w/v) BSA, the Biotin-Cap-PE molecules in the virus envelope were labeled in 0.01 mg mL^{-1} Alexa-Fluor 660-conjugated-streptavidin (Alexa-Fluor 660-SA, Molecular Probes) solution. The excessive SYTO 82 and Alexa-Fluor 660-SA were washed out by 1× PBS, respectively. The as-prepared slides were observed with a fluorescence confocal microscope (Andor Revolution XD). The SYTO 82 and Alexa-Fluor 660 were excited by laser at 561 nm with a 605 nm filter (595−615 nm) and at 640 nm with a 685 nm filter (665−705 nm), respectively. All the images were collected and processed with Andor iQ (Andor Technology PLC.).

Capture of Biotinylated PrV by Biotin-SA-Magnetic Beads. Twenty microliters of SA-PEG-magnetic beads (see Supporting Information), 10 μL of biotinylated PrV (10^9 pfu mL^{-1}), and 10 μL of PBS were mixed and incubated for 30 min, so that the biotinylated PrV virions were sufficiently bound to the beads. Then the complex was magnetically captured and rinsed three times with 1× PBS. The magnetically separated supernatant and final precipitate were detected by PCR to identify the captured virus. Control experiments were conducted on common PrV mixed with SA-PEG-magnetic beads and biotinylated PrV mixed with biotin-blocked SA-PEG-magnetic beads, respectively.

One-Step Growth Assays. PrV without and with biotin label were inoculated in Vero cells at a multiplicity of infection (MOI, ratio of infectious virus particles to cells) of 3, respectively. After 1 h of adsorption at 4 °C, the inoculum was replaced by prewarmed DMEM medium containing 2% FBS. Then viruses were allowed to infect normal Vero cells for 90 min at 37 °C. Then, remaining extracellular virus was inactivated by citrate solution (40 mM sodium citrate, 10 mM potassium chloride, and 135 mM sodium chloride, pH 3.0). Subsequently, the cells were incubated in DMEM supplemented with 2% (w/v) FBS. Supernatants were harvested immediately thereafter (0 h) or after 2, 4, 8, 12, 16, 20, 24, 36, 48, and 64 h of incubation at 37 °C. Virus progenies were titrated on normal Vero cells according to a previous report.[27]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting information and supplementary movie. This material is available free of charge via the Internet at http://pubs.acs.org.

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