

# Polystyrene nanoparticles induce stronger cellular and more durable humoral immune responses against an inactivated bovine herpesvirus 1 isolate

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# Abstract

The inactivated bovine herpesvirus type 1 (BoHV-1) vaccines are generally safe and suitable for use in dairy and pregnant cattle, but induces weaker cellular immune responses and shorter antibody responses compared with the modified live virus vaccine. In this study, we used polystyrene (PS) nanoparticles (100 nm) as a carrier for purified inactivated broken BoHV-1 to improve cellular and humoral immune responses compared with the traditional inactivated vaccine. Mice were injected intramuscularly with the inactivated complex mixed with ISA206 adjuvant. Transmission electron microscopy showed that the PS nanoparticles displayed broken BoHV-1 on their surfaces. After validation of BoHV-1 and gB gC gD gE tegument proteins, it proved that the BoHV-conjugated PS nanoparticles induced higher-titer and more durable antibody responses. The inactivated BoHV-PS nanoparticle complex elicited neutralizing antibodies (titer  $\sim 2^6$ ) in 5 weeks post-immunization in mice. The CD4/CD8 ratio was higher in mice immunized with PS nanoparticles compared with other groups. However, this ratio reached its maximum 1 week later than in mice immunized with ISA206+BoHV-1 or BoHV-1. Levels of interleukin (IL)-4, IL-6, and interferon- $\gamma$  in followed similar patterns. In conclusion, this pilot study demonstrated that PS nanoparticles can adjuvant inactivated BoHV-1 vaccines, enhancing both cell-mediated immune responses and the duration of antibody responses. This study provides the foundation for a new development platform for inactivated vaccines, which can elicit potent cellular and humoral immune responses in animals and humans.

## Introduction

Bovine herpesvirus type 1 (BoHV-1) is a major cause of bovine respiratory diseases and other immunosuppressive diseases such as conjunctivitis, infectious bovine rhinotracheitis, balanoposthitis

and abortion (Afroz et al. 2019). The BoHV-1 genome encodes more than 70 proteins (Jefferson et al. 2018) including 11 tegument proteins (gB, gC, gD, gE, gG, gH, gI, gL, gK, gM, and gN), all of which except for gN are glycoproteins (Pires de Mello et al. 2016). These glycoproteins are major targets of immune cells. The gB, gC, gD and gE envelope glycoproteins were reported to be most abundant in the viral tegument layer (Kaur and Chandra 2016). Therefore, these glycoproteins were chosen to develop subunit vaccines or diagnostic reagents (for instance, the gB protein was used by IDEXX).

Both modified live virus (MLV) vaccines and inactivated vaccines can prevent invasion of BoHV-1. However, the MLV vaccine [gE (US8)-deleted or gE-TK-deleted BoHV-1] induced stronger cellular immune responses than the inactivated vaccine. Among pregnant and dairy cattle, the MLV vaccine can induce more serious tissue lesions, including ovarian lesions that may lead to abortion, compared with the inactivated vaccine (Chase et al. 2017). For these reasons, we hypothesized that an adjuvant might improve cell-mediated immune responses against the inactivated BoHV-1 vaccine. To this end, we found inspiration in nanomaterials, as it was previously reported that silica nanoparticles could improve the magnitude and duration of T cell responses (Cheng et al. 2017; Mahony et al. 2014; Mody et al. 2015; Mody et al. 2014; Mody et al. 2013).

As a non-degradable material, we hypothesized that polystyrene (PS) nanoparticles may have similar effects on immune responses. Generally, PS nanoparticles have been used as a model system to test the carrier effects of proteins and peptides due to their good biological compatibility and safety in cells and animals. Other advantages of PS nanoparticles include ease of preparation and modification of chemical groups, uniformity of diameter, and smooth surfaces. These advantages make PS nanoparticles ideal carriers for proteins and peptides in vitro and in vivo. Naked 175 nm PS nanoparticles induced secretion of cytokines [such as interleukin (IL)-4, IL-1 $\beta$ , IL-6 and tumor necrosis factor- $\alpha$ ] in human peripheral blood mononuclear cells. They also induced immune responses mediated by CD4<sup>+</sup> cells, CD86<sup>+</sup> cells, and dendritic cells (DCs) (Frick et al. 2012). A 100-nm PS nanoparticle was also tested for uptake by human macrophages and monocytic cell lines (Oleg Lunov et al. 2011). The authors found that the nanoparticles could be taken up by macrophages, and that the optimal particle size for uptake was between 250 nm and 3  $\mu$ m (Oleg Lunov et al. 2011). Johrden et al. compared carboxyl-modified PS beads (24, 60, 93, 220 and 340 nm in diameter) with adenovirus as carriers for ovalbumin (OVA). They found that following immunization with equivalent protein amounts, the antibody responses in PS nanoparticle-immunized animals were lower than those of adenovirus-immunized animals, but higher than those of animals immunized with soluble OVA. This pattern was the same irrespective of particle size. The authors demonstrated that the use of nanoparticles as carriers allowed the reduction of antigen dose to levels similar to immunization with soluble protein, and induced strongly enhanced antibody responses (Lena Johrden1 and Oliver Wildner1 2013). Kumar et al. compared different shapes and sizes of PS nanoparticles as carriers for OVA in vivo and in vitro. They found that spherical nanoparticles (193 nm in diameter) induced Th1 biased responses. Spherical nanoparticles smaller than 193 nm induced both Th1 and Th2 responses (Kumar et al. 2015). Rod-shaped nanoparticles (376 nm and 1530 nm in length) also

induced Th2 responses. Based on these data, we hypothesized that PS nanoparticles could be used as carriers for inactivated veterinary vaccines to improve the immune responses.

## Materials And Methods

### Expression of gB, gC, gD, and gE tegument proteins in MDBK cells

To validate the humoral immune responses exactly, BoHV-1 and eukaryotic gB gC gD gE tegument protein had been prepared as follow. The full-length *gB*, *gC*, *gD*, and *gE* genes (NC001847, GenBank) containing signal peptides and restriction sites were synthesized by T SingKe Biological Technology, Ltd. (Beijing, China). The gB, gC, gD and gE genes were cloned into the pcDNA4-*Myc*-hisB plasmid (Thermo Fisher, USA).

Madin-Darby Bovine Kidney (MDBK) cells (purchased from china institute of veterinary drug control, IVDC Beijing, China) were transfected with the eukaryotic expression vectors (pcDNA4.0-*gB*-his, pcDNA4.0-*gC*-his, pcDNA4.0-*gD*-his, pcDNA4.0-*gE*-his) using a DNA Transfect Kit (Enriching Biotechnology, Shanghai, China). MDBK cells were cultured 48 h before the medium was exchanged for selective medium [Dulbecco's modified Eagle's medium (DMEM) containing 400 µg/mL Zeocin and 10% fetal bovine serum (FBS) (BI, Israel)]. After 48 h, the proteins were validated by indirect immunofluorescence assay (IFA) with rabbit anti-BoHV-1 polyclonal antibody. The tegument proteins were secreted to the culture guided by the signal peptides in the sequences and were purified using a Mag25K/NTA Ni protein purification kit (Enriching Biotechnology, Shanghai, China). Protein purity was assessed using SDS-PAGE and Western blotting (his tag).

### Preparation of virus

BoHV-1 was purchased from the China Institute of Veterinary Drug Control. MDBK cells were inoculated with BoHV-1 in DMEM containing 10% FBS. After 48 h, the cells were centrifuged at 12,000×g for 10 min and supernatants were collected. The supernatants (600 mL) were centrifuged at 100,000×g for 30 min to pelleted BoHV-1. The precipitate (containing broken BoHV-1) was resuspended in phosphate-buffered saline (PBS) and the medium was discarded. Viral concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher, USA) and normalized to 2 mg/mL.

### Preparation of PS nanoparticles conjugated to BoHV-1

The 100-nm PS nanoparticles were purchased from Enriching Biotechnology Co., Ltd. (Shanghai, China). The PS nanoparticles were diluted 10-fold in PBS, pH 6.0, and activated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1 mg/mL) and N-hydroxysuccinimide (5 mg/mL) at 37°C for 15 min. The activated PS nanoparticles were centrifuged for 20 min at 13,000×g and the supernatant was discarded. The nanoparticles were resuspended in 0.5 mL of PBS, pH 6.0, and subjected to ultrasonic vibration for 5 min in an ultrasonic cleaner. BoHV-1 (10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 µg) was conjugated to 1 mL of activated nanoparticles at 37°C for 1 h to determine the optimal

conjugation ratio. BoHV-1 remaining in supernatants was measured by BCA assay after centrifugation. The optimal conjugation ratio was 50 µg BoHV-1 per 1 mL of diluted nanoparticles. The conjugated complex (PS nanoparticles+BoHV-1) was resuspended in 500 µL of PBS, pH 7.0, and subjected to ultrasonic vibration for 5 min in an ultrasonic cleaner. Finally, the complex was inactivated with 0.2% formaldehyde at room temperature for 72 h (Hernando Duque\* and Letchworth\* 1989).

### **Observation of PS nanoparticles conjugated to BoHV-1 by transmission electron microscopy (TEM)**

The conjugated complex and purified BoHV-1 were diluted 10-fold in PBS, pH 7.4, and dried onto carbon-coated copper grids prior to observation. The size and morphology of PS nanoparticles were determined by field emission gun-transmission electron microscopy (FEG-TEM, JEOL JEM 2100F, Japan) at an operational voltage of 200 kV. The diffraction ring patterns, lattice fringes, and d-spacing were examined in high resolution mode.

### **Preparation of inactivated vaccine**

MONTANIDE™ ISA 206 VG (5 mL warmed to 31±1°C) was agitated at 350 *g*/min for 5 min and added to 5 mL of inactivated BoHV-1/PS nanoparticle complex. The mixture was agitated for 5 min at 350×*g* and placed at 20°C for 1 h with minimal transport and agitation.

### **Animals and vaccination procedure**

Female specific pathogen-free Kunming mice (8 weeks old) were purchased from SPF Biotechnology Co., Ltd. (Beijing, China) and housed in individually vented rodent caging systems at the Heilongjiang Bayi Agricultural University (Daqing, China). Animal procedures were approved by the University of Heilongjiang Bayi Agriculture University Institutional Animal Care and Use Committee in accordance with the mandates of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), AAALAC International, and the Public Health Service Office of Laboratory Animal Welfare. Three hundred mice were randomly allocated to one of six groups (PBS, PS nano, in-BoHV-1, PS+BoHV-1, ISA206+BoHV-1, ISA206+PS +BoHV-1). Mice were injected twice with 50 µg of BoHV-1 into the quadriceps femoris muscle at 4-week interval. The control groups were injected with equal volumes of diluted PS nano or PBS alone. Five mice were narcosis using ketamine (10 mg/kg) during vaccination and blood collection by enucleating the eyeball, and the blood was collected in heparin sodium anticoagulant tubes for flow cytometry assays. Blood (200 µL) was sampled from another five mice via the tail vein and collected in 1.5 mL Eppendorf tubes. After clotting, serum was used for enzyme-linked immunosorbent assays (ELISAs).

### **Flow cytometry analysis of CD4+ and CD8+ T cells**

Peripheral blood containing anticoagulant was lysed with 2 mL of red blood lysis buffer, gently mixed immediately by pipetting, and allowed to stand for 5 min at room temperature. Next, the blood was centrifuged (400×*g* for 5 min). Lymphocytes were collected and washed twice with PBS, pH 7.4, then

resuspended in 100  $\mu$ L of sterile PBS. The lymphocytes were stained with 1  $\mu$ L of anti-CD4 monoclonal antibody [fluorescein isothiocyanate (FITC)-conjugated] and 1  $\mu$ L of anti-CD8 monoclonal antibody [phycoerythrin (PE)-conjugated; BD Bioscience] in the dark. The stained lymphocytes were washed twice and resuspended in 200  $\mu$ L of PBS (Slebioda et al. 2017). Finally, the frequencies of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells were assessed using a flow cytometer (Beckman Coulter, USA).

### **ELISA to detect total IgG binding to gB, gC, gD, gE protein and BoHV-1**

The ultracentrifuged BoHV-1 and gB, gC, gD, and gE proteins (2.1) expressed in MDBK cells (validated by indirect IFA using rabbit anti-BoHV-1 polyclonal antibody purified by protein A chromatography) were coated in 96-well ELISA plates (Corning, USA) at concentrations of 1  $\mu$ g/mL (Batra et al. 2017). The plates were blocked overnight at 4°C with 1% bovine serum albumin (Amerisco, USA). The collected serum was diluted 300-fold in PBS, pH 7.4, containing 0.1% Tween-20 for BoHV-1 and 100-fold for gB, gC, gD, and gE proteins. The plates were incubated for 1 h at 37°C and washed three times. Horseradish peroxidase (HRP)-labeled secondary antibody (100  $\mu$ L) was added and incubated for 45 min at 37°C. The wash step was repeated. Trimethylbenzidine (TMB) (100  $\mu$ L) (Solarbio Life Science, Beijing) was added to each well and incubated for 15 min at room temperature. Finally, the reaction was stopped with 50  $\mu$ L of 2M H<sub>2</sub>SO<sub>4</sub>, and absorbance at 450 nm was measured using a microplate reader (BioTEK ELx800 USA).

### **Detection of virus-neutralizing antibody**

Inactivated serum (25  $\mu$ L) obtained 5 weeks post-immunization was diluted (1:8, 1:16, 1:32, 1:64 and 1:128) and mixed with 100 tissue culture infectious doses (TCID<sub>50</sub>) of BoHV-1 (25  $\mu$ L) in DMEM. The mixture was incubated for 1 h at 37°C to neutralize the virus, then added to monolayer MDBK cells. Cells were observed for the appearance of cytopathic effect for 7 days (Chung et al. 2016). Titers of neutralizing antibodies were calculated using the Karber method (Edens et al. 2015).

### **ELISAs to determine serum IL-4, IL-6, and interferon (IFN)- $\gamma$ levels**

IL-4, IL-6, and IFN- $\gamma$  cytokine ELISA kits were purchased from Proteintech Co., Ltd. (USA). The protocol below was followed to determine levels of IL-4, IL-6, and IFN- $\gamma$  in the serum collected in 2.7. The sera and 10-fold diluted standards (25  $\mu$ L) were pipetted into wells containing 75  $\mu$ L of sample dilution buffer and incubated for 120 min at 37°C. The plates were washed and spun dry four times. Diluent antibody solution (100  $\mu$ L) was transferred to the wells and incubated for 2 h at 37°C. After washing, diluent HRP solution (100  $\mu$ L) was added to wells and incubated for 20 min. After a final wash, TMB substrate was added to wells and incubated for 15 min. The reaction was stopped using stop solution (50  $\mu$ L). Absorbance was measured at 450 nm and 630 nm immediately (BioTEK ELx800 USA).

### **Statistical analyses**

Results were analyzed by one-way analysis of variance and differences between groups were assessed using Tukey's honestly significant difference test in GraphPad Prism 7.0.

# Results

## The gB, gC, gD, and gE tegument proteins were expressed in MDBK cells

Double digestion of recombinant plasmids showed that the eukaryotic expression vectors were constructed correctly.

SDS-PAGE showed that the gB, gC, gD, and gE proteins had similar molecular weights (about 180 kDa and 60 kDa) but that two protein bands were present, which were unexpected (Figure 1A). The IFA confirmed that the gB, gC, gD, and gE proteins were expressed successfully and maintained natural structure in MDBK cells validated by rabbit anti-BoHV-1 polyclonal antibody (Figure 1B).

## PS nanoparticles conjugated with BoHV-1 were observed by TEM

By TEM, the naked 100 nm PS nanoparticles were monodisperse (Figure 2A) and approximately 95–110 nm in diameter prior to conjugation with BoHV-1. The ultra-centrifuged BoHV-1 was about 50 nm (Figure 2C), comparing with other standard BoHV-1 (about 100 nm) (Figure 2D). The diameter increased to approximately 150 nm after conjugation with BoHV-1 (Figure 2B). Because the BoHV-1 was broken by 100,000×*g* centrifugation. This result indicated that BoHV-1 was successfully conjugated to PS nanoparticles (Figure 2B).

## Total IgG responses against gB, gC, gD, and gE proteins and BoHV-1 determined by ELISA

Antibody titers against BoHV-1 reached their maximum 3 weeks post-immunization, then decreased slowly, persisting for more than 10 weeks. Although antibody titers in the PS+ISA206 group rose later than in the ISA206 group, they also decreased more slowly than in the ISA206 group, thus resulting in a longer duration of the humoral immune response (Figure 3A). Antibody titers against the gC protein (Figure 3C) reached their maxima 3 weeks post-immunization, 1 week earlier than antibody titers against the gB, gD and gE proteins (Figure 3B, D and E). Judging from the absorbance at 450 nm, antibody titers against the gD protein were higher than those against other proteins from weeks 3 to 5 post-immunization (Figure 3D), and persisted as long as those against other proteins. Comparing the antibody titers against the gB protein in different groups showed that the titers of the PS+ISA206+BoHV-1 and ISA206+BoHV-1 groups were significantly different, with the former reaching its maximum 5 weeks post-immunization, 1 or 2 weeks later than for the gC, gD, and gE proteins.

BoHV-1-neutralizing antibody titers in the PS+ISA206+BoHV-1 group ( $\sim 2^6$ ) were higher than in the other groups 5 weeks post-immunization. The neutralizing titers in the PS+BoHV-1 group were similar to those of the ISA206+BoHV-1 group (Figure 3F).

## Measurement of cellular immune responses by flow cytometry and ELISA

Based on the absorbance at 450 nm, secretion of IL-4 was induced by ISA206 and BoHV-1, but naked PS nanoparticles could not induce significant production of IL-4 (Figure 4A).

The IL-6 levels in the PS+ISA206+BoHV-1 group reached their maximum at 5 weeks post-immunization, two weeks delayed compared with the ISA206+BoHV-1 group but with higher maximum levels (Figure 4B). Naked PS nanoparticles also did not induce secretion of IL-6. Levels of serum IFN- $\gamma$  reached their maxima 3 weeks post-immunization in the ISA206+BoHV-1 group, weeks earlier than in the PS+ISA206+BoHV-1 group but with a lower maximum (Figure 4C). Naked PS nanoparticles induced low-level secretion of IFN- $\gamma$ , by contrast with IL-4 and IL-6. Serum cytokine levels in the PS and PS+ISA206+BoHV-1 groups were higher and persisted longer than levels in the PS+BoHV-1 and PS groups.

The CD4/CD8 ratio was highest in the PS+ISA206 group, followed by the PS+BoHV-1 and PS nanoparticles group, and then by the ISA206+BoHV-1 and In-BoHV-1 groups. The CD4/CD8 ratio in the PS+ISA206+BoHV-1 group reached a higher maximum value 1 week earlier compared with the PS+BoHV-1 group (Figure 4D). The CD4/CD8 ratios of groups that received PS nanoparticles were higher than those of groups that did not receive nanoparticles. This finding indicated that the ISA206 adjuvant alone was unable to induce strong T cell responses.

## Discussion

As we hypothesized, PS nanoparticles acted as an adjuvant to conjugated BoHV-1, inducing stronger T cell responses and more durable antibody responses than ISA206 adjuvant. This work forms the basis of a developmental platform for potentiating cell-mediated and humoral immune responses against inactivated vaccines.

The CD4/CD8 ratio reached its maximum 5 weeks post-immunization, then decreased slowly until 10 weeks. The CD4/CD8 ratio of the PS + ISA206 + BoHV-1 group was higher than that of other groups. The increase in CD4<sup>+</sup> T cell frequency suggests that the PS nanoparticles induced Th 1 cell differentiation (Frick et al. 2012). It was reported that optimal vaccine-induced protective immunity depends on contributions from both CD4<sup>+</sup> T cells and antibodies (Farris et al. 2010). Frick et al. showed that functionalized PS nanoparticles triggered human DC maturation resulting in enhanced CD4<sup>+</sup> T cell activation (Frick et al. 2012). The PS nanoparticles also have been taken up by immune cells resulting in BoHV-1 delivery to both MHC class I and MHC class II antigen presentation pathways, resulting in further CD4<sup>+</sup> T cell activation (Stano et al. 2012). Elevated serum levels of IL-4 and IL-6 also suggested that Th2 cells had differentiated in response to immunization. To better understand the mechanisms underlying cellular immune responses to PS nanoparticles conjugated to BoHV-1, more data will be needed at the level of T cells and other immune cells. However, our results suggest that the PS nanoparticles were safer than ISA206. Because IL-4 is associated with toxicity, increased secretion of IL-4 may signify an ongoing inflammatory reaction (Gui et al. 2011; Park et al. 2010). Comparing the PS + ISA206 + BoHV-1 group with others, alterations in IFN- $\gamma$  levels indicated that cellular immune responses had been enhanced. Judging

from the results above, the improved cell responses were primarily due to PS nanoparticles, because levels of IFN- $\gamma$  in the PS + ISA206 + BoHV-1 and PS nano groups were higher than those in the ISA206 + BoHV-1 group. Secretion of IFN- $\gamma$  indicated that Th1 cells were activated and that cellular immune responses had been initiated (Anne et al. 2018). Secretion of IL-4 and IL-6 signified that Th2 cells were activated and that antibody was being produced (LA. Babiuk \* 1996).

To validate the humoral immune responses, BoHV-1 was purified by ultracentrifugation and the gB, gC, gD, and gE proteins were expressed in MDBK cells. The TEM results show that BoHV-1 was broken up after ultracentrifuged by 100,000  $\times$  g for 30 min (Fig. 2C & D)(Barber et al. 2017). The gB, gC, and gD proteins had been validated as subunit vaccine candidates and conferred significant protection from BoHV-1 challenge when formulated with an effective adjuvant(\* 1997; Blanc et al. 2012; J. CHARLES WHITBECK 1988; Keil et al. 2005; Keil et al. 2010; Levings et al. 2015; M.J. Kaashoek"" and Oirschot\*\$ 1998). The gE protein could also elicit neutralizing antibodies, although it was a virulence factor of BoHV-1 (van Drunen Littel-van den Hurk 2006). Interestingly, we found that the gB, gC, gD and gE proteins had similar molecular weights (about 130 ~ 180 kDa and 60 kDa) by SDS-PAGE and Western Blotting (his tag) (Fig. 2A). This result was unexpected (Fig. 1A) based on the theoretical molecular weights of these proteins (gC 55.4 kDa, gD 44.9 kDa, gE 26.9 kDa; Uniprot.org). Chowdhury et al. reported the molecular weight of gC as 90 kDa, when it assembled into homodimers (\* 1997). Keil et al. showed that the molecular weight of gB was 130 kDa, until it was cleaved into a 72-kDa N-terminal subunit and a 55-kDa C-terminal subunit (Keil et al. 2005). The molecular weight of the gE protein (55 kDa, 103 kDa, and 137 kDa) differed based on its levels of phosphorylation (Shaw et al. 2000). The molecular weight of native gD protein was 72 kDa. However, the apparent molecular weight of the gD protein expressed by MDBK cells was about 130 kDa, significantly different from its theoretical molecular weight (XIAOPING LIANG 1995) (S. VAN DRUNEN LITI-EL-VAN DEN HURK 1995). After comparing the SDS-PAGE and Western Blotting, it was found that the bands at 130 kDa-180 kDa was the proteins, and the bands at 60 kDa were not the tegument proteins. The IFA (Fig. 1B) results showed that the recombinant proteins were bound by an anti-BoHV-1 polyclonal antibody, indicating that the proteins were expressed and maintained their native structure.

The humoral immune response against BoHV-1 was improved by its conjugation to PS nanoparticles. PS nanoparticles conjugated with BoHV-1 not only improved secretion of antibody, but also lengthened the duration of antibody responses. One explanation may be that BoHV-1 was conjugated with PS nanoparticles via chemical bonds, and BoHV-1 was released gradually and degraded. Antibody titers against the gC protein reached their maximum by week 3 post-immunization, one week earlier than other proteins. One explanation for this result may be that gC binds heparan sulfate proteoglycan, making it easily recognized by antigen-presenting cells (Jones and Chowdhury 2007). The generation of anti-gC antibodies needs to be explored further in cattle, as it may mirror that in mice. If so, the gC protein could represent a target for early diagnosis of BoHV-1 infection. Antibody titers against gB protein reached their maximum 5 weeks post-immunization, one week later than the gD and gE proteins. The antibody titers against the gD protein confirmed the conclusion that the gD protein expressed by eukaryotic cells could be used as a diagnostic target (Brewer et al. 2017). Usually, the gB protein has been used to assess BoHV-

1 molecular evolution (Hidayati et al. 2018). It was reported that the gE gene was a duplicated non-essential gene, but it also could bound to receptors on cell membranes, and induced viral invasion of the trigeminus (M.J. Kaashoek and Oirschot 1998). The generation of antibody titers against the gE protein meant that the gE protein could be recognized by immune cells, and induced immune responses. Antibodies against BoHV-1, gB, gC, and gD persisted for more than 10 weeks. The OD450 values for serum antibody were above 0.4 for the PS nano + ISA206 + BoHV-1 group, signifying that immune cells were activated for prolonged durations.

The BoHV-1 neutralizing antibody titers 5 weeks post-immunization indicated that PS nanoparticles displayed BoHV-1 on its surface successfully, and elicited neutralizing antibodies to the same extent as a traditional inactivated vaccine (titer  $\sim 2^6$ ) (Chung et al. 2016). Protection of cattle against BoHV-1 challenge (3 mL,  $10^{7.0}$  TCID<sub>50</sub>) required neutralizing antibody titers above  $2^3$  (Chung et al. 2016).

The enhanced cell and humoral immune responses in the PS + ISA206 + BoHV-1 group were a combined function of BoHV-1, ISA206 adjuvant and PS nanoparticles. The mechanisms may be as follows. The nanoparticles can deliver antigen to DCs for cross-presentation to antigen-specific T cells (Rietscher et al. 2016). Nanoparticles can be efficiently engulfed by DCs and induce DC maturation and cytokine release, activating antigen-specific immune responses and enhancing T cell proliferation, IFN- $\gamma$  production and generation of cytotoxic T lymphocytes (Dudek et al. 2018). The traditional adjuvant ISA206 is a water-oil-water emulsion, which may maintain the native structures of antigen better than water-oil adjuvants (Fuentelba et al. 2019; G.F. El-Bagoury and Darwish 2010; Zhugunissov et al. 2018).

In this study, we showed that PS nanoparticles could improve the strength of cell-mediated immune responses and the duration of humoral immune responses against an inactivated BoHV-1 vaccine. Non-biodegradable nanomaterials (PS, silica, gold, silver, etc.) have been applied in diagnostic fields and for drug delivery (Li et al. 2019; Mody et al. 2014; Mody et al. 2013) because of their biocompatibility and their diverse shapes and diameters. Injection of non-biodegradable nanomaterials may be unsafe when animal products (such as meat or milk) are consumed by humans. Therefore, selecting biodegradable nanoparticles to replace the PS nanoparticles will be our ultimate aim. Biodegradable nanomaterials [e.g., dextran, poly (lactic-co-glycolic acid), chitosan, polyethylene glycol, pullulan, and peptides] have been applied in drug delivery and cancer therapy because of their safety and degradation characteristics in vivo (Brewer et al. 2017; Gheibi Hayat and Darroudi 2019; Ho et al. 2018; Hong et al. 2017; Jiang et al. 2013; Liang et al. 2019; Mona Gupta 2004; Xiaobo Fan et al. 2016). Thus, these materials may be candidates for future research.

In conclusion, we showed that PS nanoparticles improved cellular immune responses and lengthened the duration of antibody responses against inactivated BoHV-1. Based on these results, it may now be possible to develop a complex inactivated BoHV-1 vaccine that induces strong cellular and humoral immune responses, which would be safe for use in dairy and pregnant cattle. And biodegradable nanoparticles will replace the PS nanoparticles is necessary in the future research for safe animal products.

## Declarations

**Authors' contributions:** Mr Xingbo Liu and Mr hongbo Ni conceived and designed research. Mr Xingbo Liu and Mr Zhihao Xin conducted experiments. Ms Fan zhang , Ms Luyao zhang, and Liwei Gu contributed new reagents or analytical tools. Ms hanyu Yan analyzed data. Mr Xingbo Liu wrote the manuscript. All authors read and approved the manuscript.

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### Compliance with ethical standards

The study was approved by the Animal Experiments Committee of the Heilongjiang Bayi Agricultural University.

### Conflict of interest statement

No potential conflict of interest.

### Ethics approval and consent to participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

### Consent for publication

Yes.

### Availability of data and material

All data and materials are true and reliable

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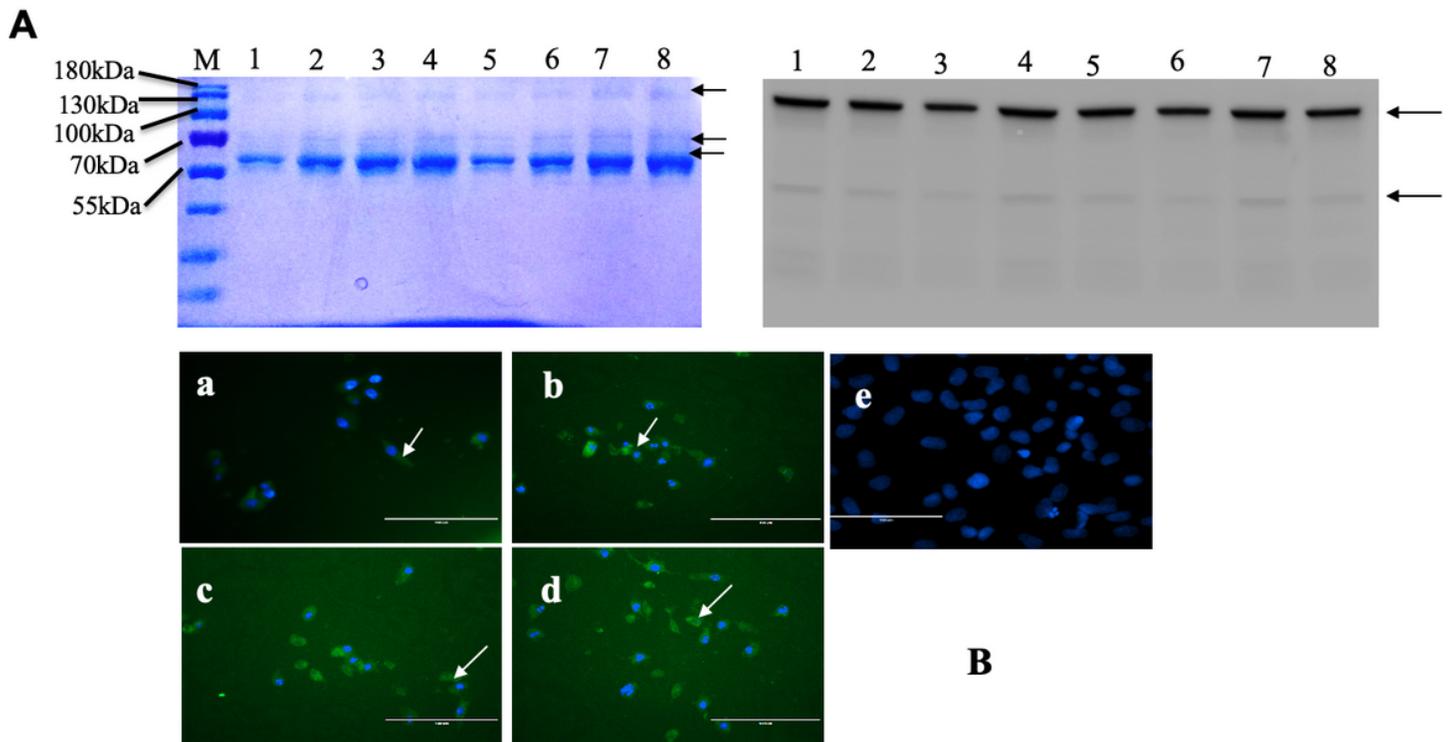
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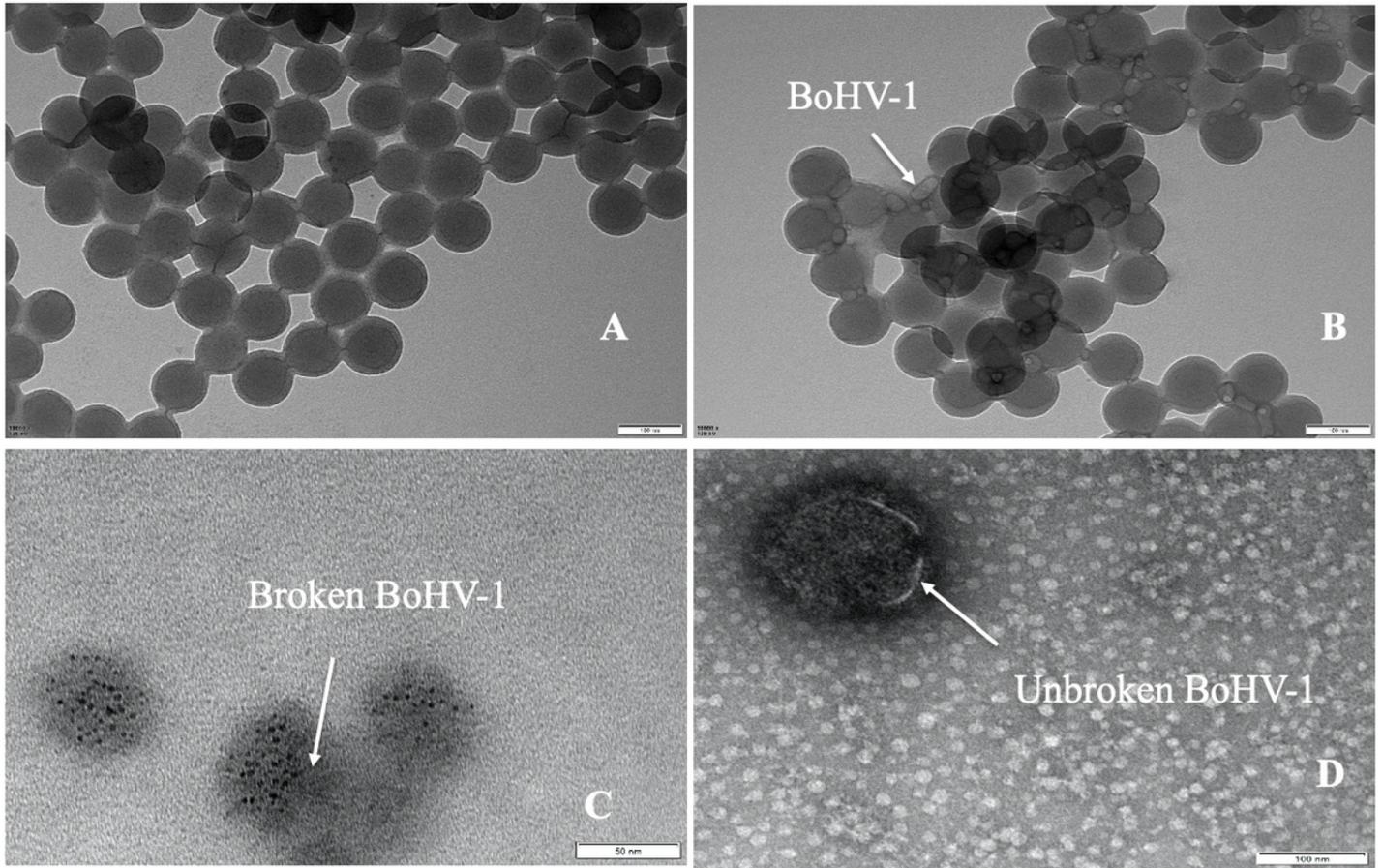
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## Figures



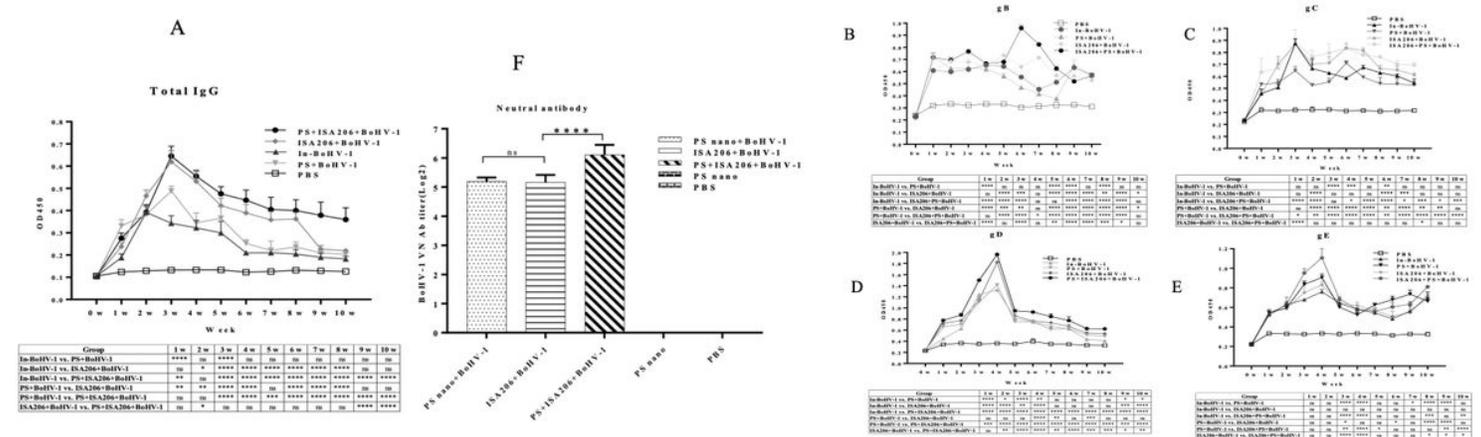
**Figure 1**

A: Assessment of gB, gC, gD, and gE proteins expressed by MDBK cells Lane M: marker; lanes 1 and 2: gB; lanes 3 and 4: gC; lanes 5 and 6: gD; lanes 7 and 8: gE. Purity identification of gB gC gD gE proteins by SDS-PAGE and Western blotting (his tag). Lane: M: marker, 1&2: gB, 3&4: gC, 5&6: gD, 7&8: gE; his tag purified proteins contained 3 molecular weights, which was different with theory, further the bands were validated by Western Blotting (his tag), the tegument proteins was 130kDa~180kDa, just thin bands at 60kDa. The molecular weights of the gB, gC, gD, gE proteins differed from those expected based on the theoretical molecular weights of the native proteins (gB:101.19 kDa, gC 55.4 kDa, gD 44.9 kDa, gE 26.9 kDa; Uniprot.org). but there were two light-colored bands in the SDS-PAGE. B: MDBK cells were transfected with pcDNA4.0-gB/gC/gD/gE-his expression vectors and protein expression were assessed using an indirect immunofluorescence assay with rabbit anti-BoHV-1 polyclonal antibody. Antigenicity validation of gB, gC, gD, gE proteins expressed in MDBK cell line by IFA with rabbit anti-BoHV-1 polyclonal antibody: a: gB; b:gC; c:gD; d:gE; e:Negative. a, b, c, d: MDBK cells were transfected with the pcDNA4.0-gB-his, pcDNA4.0-gC-his, pcDNA4.0-gD-his, and pcDNA4.0-gE-his plasmids, respectively, and expression of the gB, gC, gD, and gE proteins (respectively) in the cytoplasm was assessed. e: MDBK cells were transfected with negative control pcDNA4.0-Myc-his plasmid.



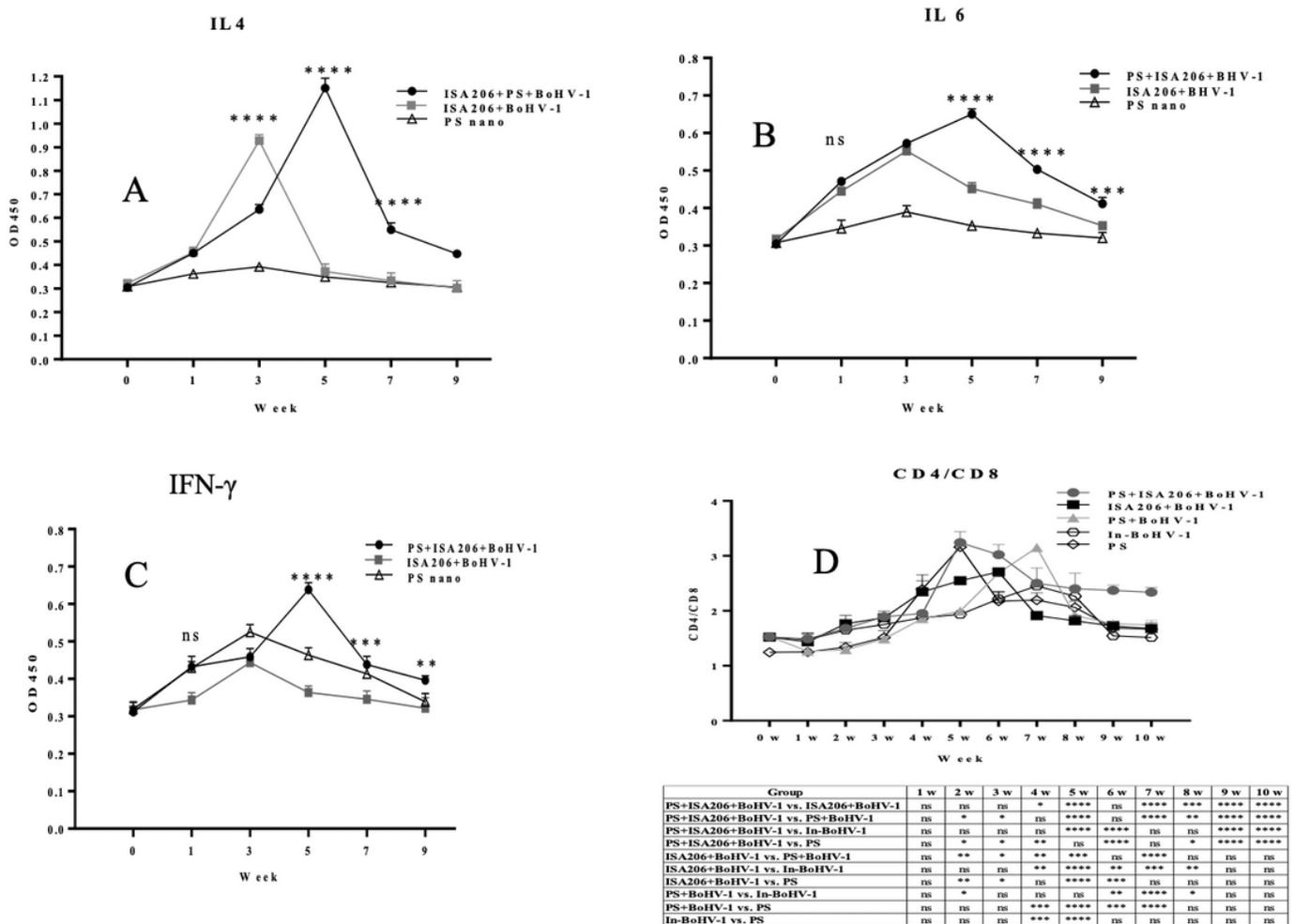
**Figure 2**

TEM of PS nanoparticles conjugated to BoHV-1 A: Naked PS nanoparticles scanned by TEM (50,000 $\times$ ). The PS nanoparticles were monodisperse and spherical in shape. B: BoHV-1 was conjugated to PS nanoparticles scanned by TEM (50,000 $\times$ ). The broken BoHV-1 was conjugated to the surface of PS nanoparticles, but did not cover it fully. C: Broken BoHV-1 [Centrifuged by 100,000 $\times$ g], no tegument layer was observed D: Unbroken BoHV-1 show a tegument layer



**Figure 3**

Total IgG against BoHV-1/gB/gC/gD/gE was detected in serum. A: BoHV-1(100ng) was coated in wells and binding of total IgG in mouse serum was assessed by ELISA. B, C, D, E: gB, gC, gD, gE proteins (100 ng) were coated in wells and binding of total IgG in mouse serum was assessed by ELISA. F: Serum microneutralization assay 5 weeks post-immunization. Serum antibody neutralization of BoHV-1 (25  $\mu$ L serum) was tested using a microneutralization assay 7 days post-immunization. Titers of neutralizing antibodies were calculated using the Karber method. Analysis of humoral immune responses showed that PS nanoparticles conjugated to BoHV-1 induced more durable antibody responses and higher neutralizing antibody titers than inactivated BoHV-1+ISA206. Asterisks indicate the magnitude of statistically significant differences between groups (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ ).



**Figure 4**

Levels of serum IL-4, IL-6, and IFN- $\gamma$  determined by ELISA and CD4/CD8 ratio determined by flow cytometry. Levels of IL-4 (A), IL-6 (B) and IFN- $\gamma$  (C) were determined in serum (25  $\mu$ L) using ELISA kits. The results showed that using PS nanoparticles as a carrier improved cellular immune responses and induced higher IFN- $\gamma$  levels compared with ISA206. D: Frequencies of CD4+ and CD8+ T cells assessed by flow cytometry. Anticoagulant-treated blood (200  $\mu$ L) was lysed with red blood lysis buffer and lymphocytes were collected and stained with anti-CD4 (FITC) and anti-CD8 (PE) monoclonal antibodies. The stained

lymphocytes were analyzed by flow cytometry. PS nanoparticles enhanced CD4+ T cell responses, irrespective of the presence or absence of ISA206 adjuvant. In the presence of ISA206, CD4+ T cell responses reached their maxima 1 week earlier than in the PS+BoHV-1 group and persisted longer. Asterisks indicate the magnitudes of statistically significant differences between groups (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ ).