Poly (lactic-co-glycolic acid) nanoparticle-based vaccines delivery systems as a novel adjuvant for H9N2 antigen enhance immune responses

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ABSTRACT Poly (lactic-co-glycolic acid) (**PLGA**) nanoparticle used as vaccine adjuvants have been widely investigated due to their safety, antigen slow-release ability, and good adjuvants activity. In this study, immunopotentiator Alhagi honey polysaccharide encapsulated PLGA nanoparticles (**AHPP**) and assembled pickering emulsion with AHPP as shell and squalene as core (**PPAS**) were prepared. Characterization of AHPP and PPAS were investigated. H9N2 absorbed nanoparticles formulations were immunized to chicken, then the magnitude and kinetics of antibody and cellular immune responses were assessed. Our results showed that PPAS had rough strawberry-like surfaces, a large number of antigens could be absorbed on their surfaces through simple mixing. Adjuvant activity of PPAS showed that, PPAS/H9N2 can induce long-lasting and high HI titers, high thymus, spleen, and bursa of fabricius organ index. Moreover, chicken immunized with PPAS/H9N2 showed a mixed high differentiation of $CD4^+$ and $CD8a^+$ T cell, and strong Th1 and Th2-type cytokines mRNA expression. Thus, these findings demonstrated that PPAS could induce a strong and long-term cellular and humoral immune response, and has the potential to serve as an effective vaccine delivery adjuvant system for H9N2 antigen.

Key words: alhagi honey polysaccharide, pickering emulsion, poly(lactic-co-glycolic acid) nanoparticles, vaccine adjuvants, immune response

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INTRODUCTION

Vaccination is one of the safest and most effective tools to prevent many infectious and chronic diseases, such as prevent avian influenza (Wang et al., 2019a). The H9N2 avian influenza virus (**AIV**), which is one subtype of Avian influenza virus, has led to severe economic losses in the global poultry industry (Gan et al., 2019; Yitbarek et al., 2019; Su et al., 2020). It has been reported that the H9N2 virus not only reduces the egg production and growth rate of chickens (Ellakany et al., 2018; Jake et al., 2018), but also may be transmitted to humans, causing harm to human health (Jake et al., 2018; Zhou et al., 2018). It is reported that both humoral and cellular immunity plays a vital role in the protection of H9N2 infection (Park et al., 2014; Umar, et al., 2016). To enhance the immunogenicity of vaccines and produce a long-term and effective cellular and humoral immune protection effect for animals, the vaccine is usually mixed with an adjuvant (Lung et al., 2020; Plotkin. and Stanley., 2014). Aluminum adjuvant, known as the conventional adjuvant, has widely applied in vaccines because it induces effective antibodies immune responses (Yue and Ma, 2015; Nazmi et al., 2017; Wang et al., 2019a). However, aluminum adjuvant has the inability to elicit the cell-mediated type-1 T cell (**Th1**) or cytotoxic T lymphocyte (**CTL**) responses (Patel et al., 2006; Xia et al., 2018; Yue and Ma, 2015). The oil emulsion adjuvant could effectively enhance immune responses for vaccines and is widely used in veterinary, but also can cause local granuloma, abscess, and other side effects, affecting the quality of livestock products (Bo et al., 2017; Giuseppe et al., 2018). Therefore, it is still necessary to develop a safe H9N2 adjuvant that can induce humoral and cellular immune responses.

Alhagi honey polysaccharide (**AHP**) extracted from alhagi honey has been demonstrated that it possesses excellent immune enhancement, and widely used as immune-stimulator (Wusiman et al., 2019a). Our previous research has been showed that encapsulation of the

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AHP and antigen in PLGA nanoparticles as vaccine adjuvants has ability to induced long lasting and strong humoral and cellular immune responses (Mir et al., 2017; Xia, et al., 2018; Wusiman et al., 2019b). However, the antigen encapsulation method will directly expose antigens to organic solvents. Compared with particles nano-adjuvant, assembled particles-oil adjuvants pickering emulsion may adsorb more antigens because of the rough strawberry-like surface area (Xia et al., 2018).

In addition, Pickering emulsions based on PLGA nanoparticles will promote the interaction between nanoparticles and cell membranes and antigen uptake by antigen-presenting cells (**APCs**) due to their large surface area and core fluidity. Additionally, PLGA nanoparticle-based Pickering emulsion will promote nanoparticles interactions with the cell membrane due to their large surface area and good flow variability. When the nanoparticles are in contact with APCs, large amounts of antigen are taken up by the APCs, and the activated APC carry a large number of nanoparticles into lymph nodes therefore it also process excellent lymph node targeting (Mir et al., 2017; Xia et al., 2018). After uptake of a large number of antigens, immune cells induced a strong cellular or humoral immune response through different ways (Mir et al., 2017; Xia et al., 2018).

In our previous research, an immunopotentiating AHP encapsulated PLGA nanoparticles (**AHPP**) delivery system was successfully designed and developed as a nano-adjuvant. However, the antigen encapsulating method limits the clinical application of the nano-adjuvant. In this study, the assembled pickering emulsion was prepared by assembling AHPP with oil-phase squalene (**PPAS**) via phacoemulsification. We hypothesized that PPAS, as a stable vaccine adjuvant, could load large amounts of antigens on the strawberry-like surface area through simple mixing and induce strong and long-lasting humoral and cell-mediated immune response by antigen slow-release effect and different antigen treatment pathways. Therefore, the morphological structures, antigen loading efficacies, in vitro antigen release behaviors, and the change of physical forms of the PPAS adjuvants were measured. PPAS vaccine delivery system surface-adsorbed with H9N2 antigen were prepared, and their immunoadjuvant activity was detected by various methods.

MATERIALS AND METHODS

Preparation of PPAS and PEI-PPAS Emulsion

AHPP nanoparticles were prepared using the double emulsion technique as previously described (Wusiman et al., 2019a). First, immunostimulatory Alhagi honey polysaccharide (**AHP**, MW 47479 Da, Carbohydrate content = 99.0%) was dissolved in water sonicated with PLGA (MW 18KDa, 75:25) dichloromethane solution to obtain the primary AHP encapsulated PLGA emulsion, then the primary emulsion was mixed and sonicated with Pluronic F68 solution to form a stable AHPP emulsion. The obtained nanoparticle emulsion was freeze-dried, and the powder was stored at −20°C. The encapsulation efficiency of AHP in the AHPP nanoparticles was about 64.09 ± 2.55%, according to our previous research (Wusiman et al., 2019c).

AHPP based Pickering emulsion (PPAS) was prepared by sonication. Briefly, 10 mg nanoparticles are fully dissolved in 1.6 mL water and sonicated with 0.4 mL the oil phase squalene (Shanghai Macklin Biochemical Technology Co., Ltd, China) at 80 W for 90 s to form a stable PPAS emulsion (Xia et al., 2018).

Morphology and Antigen Adsorption Characteristics of the PPAS

Cryo-scanning electron microscopy (Cryo-SEM, FEI quanta250) and Scanning electron microscopy (SEM, Model S-4800 II FESEM,) were used to observed the morphology and structure of PPAS and AHPP, respectively. To evaluate the antigen loading characteristics of PPAS, OVA-FITC (green, Beijing Solaibao Technology Co. Ltd, China) solution was mixed the PPAS (red) emulsion, to prepare the OVA-FITC loaded PPAS. The morphology of antigen adsorption of PPAS was observed by confocal laser scanning microscopy.

Assay on Characterization of the PPAS

To investigate the change of the characteristics of PPAS and AHPP emulsion before and after antigen loading. The changes of polydispersity index (**PDI**), hydrodynamic size, and zeta potentials was measured using a Nano ZS instrument (Hydro2000Mu, Malvern Instruments, UK) before and after loading H9N2.

Antigen Adsorption Efficiency and In Vitro Release of PPAS

PPAS or AHPP were (5 mL, 10%, v/v) mixed with H9N2 antigen (5 mL) at room temperature for 20 min, to prepare the H9N2 loaded PPAS (PPAS/H9N2) or H9N2 loaded AHPP (AHPP/H9N2). In brief, free H9N2 in emulsion were isolated from PPAS or AHPP by means of a Sephadex G-100 column (Beijing Solaibao Technology Co. Ltd), and the antigen adsorption efficiency (**AE**) was measured with BCA assay (Beijing Solaibao Technology Co. Ltd). The H9N2 antigen adsorption efficiency was calculated using Eqs:

$$\text{Antigen adsorption efficiency} = \frac{\text{Total antigen} - \text{Supernatant antigen}}{\text{Total antigen}}$$

To determine the antigen slow effect of PPAS and AHPP, 30 mL of PPAS/H9N2 or AHPP/H9N2 were poured into the dialysis bag, and shacked at 37°C, 100 rpm. At predetermined intervals 500 μL samples were collected and the free H9N2 was separated by Sephadex G-100 column and determined by BCA assay.

Animal Immunization

Inactivated H9N2 antigen was a gift from the Animal Health and Epidemiology Center (Qingdao, China). H9N2 AIV was a reassortant rGX55 virus, which carries the HA and NA genes of A/chicken/Guangxi/55/2005 (H9N2) virus and the internal genes of PR8, was recovered by using reverse genetics. EID50/mL = $1 \times 10^{-9.75}$. H9N2 antigen was collected from allantoic fluid of chicken embryo which cultured with H9N2 virus, and then inactivated at 37°C for 24 h mixed with formaldehyde (0.1%). Animal experiments were conducted in strict accordance with the guide for the care and use of laboratory animals, Nanjing Agricultural University IACUC (Approval ID: 2019034). One-day-old HY-LINE Variety Brown chickens purchased from Hai 'an Shuangli Hatch Poultry farm (Nantong, China) were randomly divided into 5 groups (n = 22) after centralized feeding for 1 wk. The chickens were intramuscular immunized with 0.2 mL of the PPAS/H9N2 (1:1 = V_{PPAS}:V_{H9N2}), AHPP/H9N2(1:1 = V_{AHPP}:V_{H9N2}), the control groups were Alum/H9N2 (Alum Adjuvant, Thermo Fisher Scientific, Waltham, MA, 1:1 = V_{Alum}:V_{H9N2}), free H9N2, and PBS. All of the chickens were sacrificed at d 7, 21, and 35 after the second immunization.

Detection of Hemagglutination Titer and Immune Organ Index

Hemagglutination (**HI**) titer of sera weas determined by hemagglutination inhibition methods at the d 7, 21, and 35 after the final vaccination according to a previous study (Wang et al., 2016). On d 21and 35 after the second immunization, chicken (n = 5) immune organs spleen, thymus and bursa of fabricius were collected, and weighed quickly. The chicken immune organ index was calculated, immune organ index = (Immune organ weight/body weight) × 100% (Wang et al., 2016).

Activation of the $CD4^+$ and $CD8^+$ T Cells

On 21 d after the second immunization, chicken splenic lymphocyte (n = 5) were collected and stained with anti-Chicken -CD3-FITC antibody, anti-CD4-PE antibody and anti-CD8A-APC antibody (Southern Biotech) according to a previous study (Bo et al., 2018). The expression of T cell differentiation was analyzed by the flow cytometer (BD Accuri C6).

QRT-PCR Analysis of Cytokines for Peripheral Blood Lymphocytes

Peripheral blood lymphocytes were collected from immunized chickens at 21 d after the final vaccination, and the mRNA expression levels of IL-2, INF-γ, IL-4, and IL-6 were measured with qRT-PCR analysis according to a previous study. Briefly, total RNA of peripheral blood lymphocytes was isolated using TRIzol reagent (Life Technologies, Rockville, MD). Reverse transcription was performed using TaKaRa Prime Script RT reagent Kit with gDNA Eraser, and real-time PCR were performed by TB Green Premix Ex Taq II (TaKaRa Co., Ltd, Nanjing, China) according to the manufacturer's instructions. The different primers of cytokine genes and reference genes (β-actin) were shown in Table 1, and the data analyses were performed using the 2-ΔΔCt method (Bo et al., 2018).

Table 1. Chicken spleen cytokine primer design.

Gene	Primers sequences 5' to 3'	Bases number
INF-γ	F: AAAACCCACTCATACCTGCTC	21
	R: GAGTGTCACATTTCCTGGAGC	21
IL-4	F: AGGTTTCCTGCGTCAAGATG	20
	R: ATGGTGCCTTGAGGGAGGTG	20
IL-6	F: GGTGATAAATCCCGATGAAGTG	22
	R: CAGAGGATTGTGCCCGAAC	19
IL-2	F: ATCTTTGGCTGTATTTCGGTAG	22
	R: GCAGAGTTTGCTGGGTGCA	19
β-actin	F: GAAGTACCCCATTGAACACGG	21
	R: GCATACAGGGACAGCACAGC	20

Histopathological Analysis

Spleen, thymus and bursa of fabricius were collected from the immunized chickens and fixed with 4% paraformaldehyde on d 35 after the second immunization (n = 3). Then, the fixed organs were performed by hematoxylin and eosin (**HE**) staining.

Statistical Analysis

Quantitative data were expressed as means ± SEM. Statistical significance was analyzed using One-way ANOVA analysis with Turkey's test. A probability value $P < 0.05$ was considered statistically significant.

RESULTS

Morphology Observation

PPAS emulsion was prepared by ultrasonic emulsification with squalene as the internal oil core and AHPP as the hydrophilic shell. As shown in Figure 1A, AHPP was smooth spherical in morphology, and the particle size was about 200 nm. The PPAS emulsion were all spherical in morphology, with a rough surface in strawberry shape and a size of about 1,000 nm (Figure 1B). The results showed that the distribution of AHPP on the surface of PPAS emulsion were uniformly and compactly.

Characterization of OVA Adsorbed PPAS

PPAS has a large specific surface area and provide a greater number of surface gaps for antigen adsorption. As shown in Figure 2, a large among of antigen evenly distributed on the surface of PPAS. The results indicated that the large among of antigens were absorbed by the special surface gaps of PPAS.

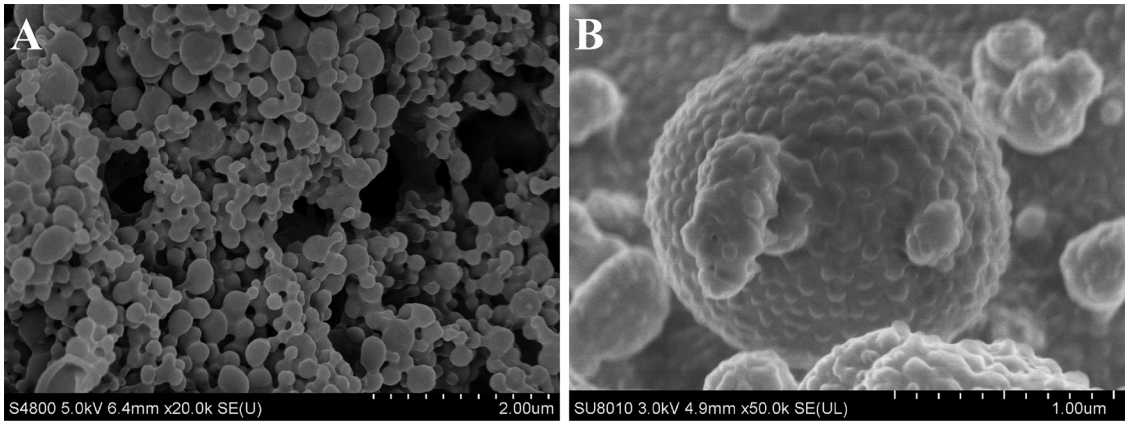

Figure 1. Morphology of the nanoparticles (A) scanning electron microscopy of AHPP; (B) cryo-scanning electron microscopy of PPAS.

Characterization of H9N2 Adsorbed PPAS

In order to detect the characterization of nanoparticles pre and post-antigen loading, the changes of size, zeta potential and PDI of the nanoparticles were measured. As shown in Figure 3, the particle size of AHPP and PPAS were about 200 nm and 1,000 nm, and the particle size of antigen loaded AHPP/H9N2 and PPAS/H9N2 were increased, in which particle size of PPAS increased more clearly. The larger particle size of PPAS/H9N2 may due to the adsorption of H9N2 antigen on the surface. The PDI value of AHPP and PPAS increased after loading antigen, but all of the value were lower than 0.3, the results that the nanoparticles have good dispersibility. The changes of zeta potentials due to the fact that a large among of negatively charge antigen adsorbed on the surface of the AHPP and PPAS.

As shown in Figure 3B, the adsorption efficiency of H9N2 antigen on AHPP and PPAS was about 20 and 65%, respectively. The in vitro release of H9N2 from AHPP/H9N2 and PPAS/H9N2 at 37°C was shown in Figure 3C. Within 12 h (pH = 7.0), more than 50% of antigen had been released from AHPP, more than 15% of antigen had been released from PPAS, and then both of the nanoparticles followed by slow release over 40 d.

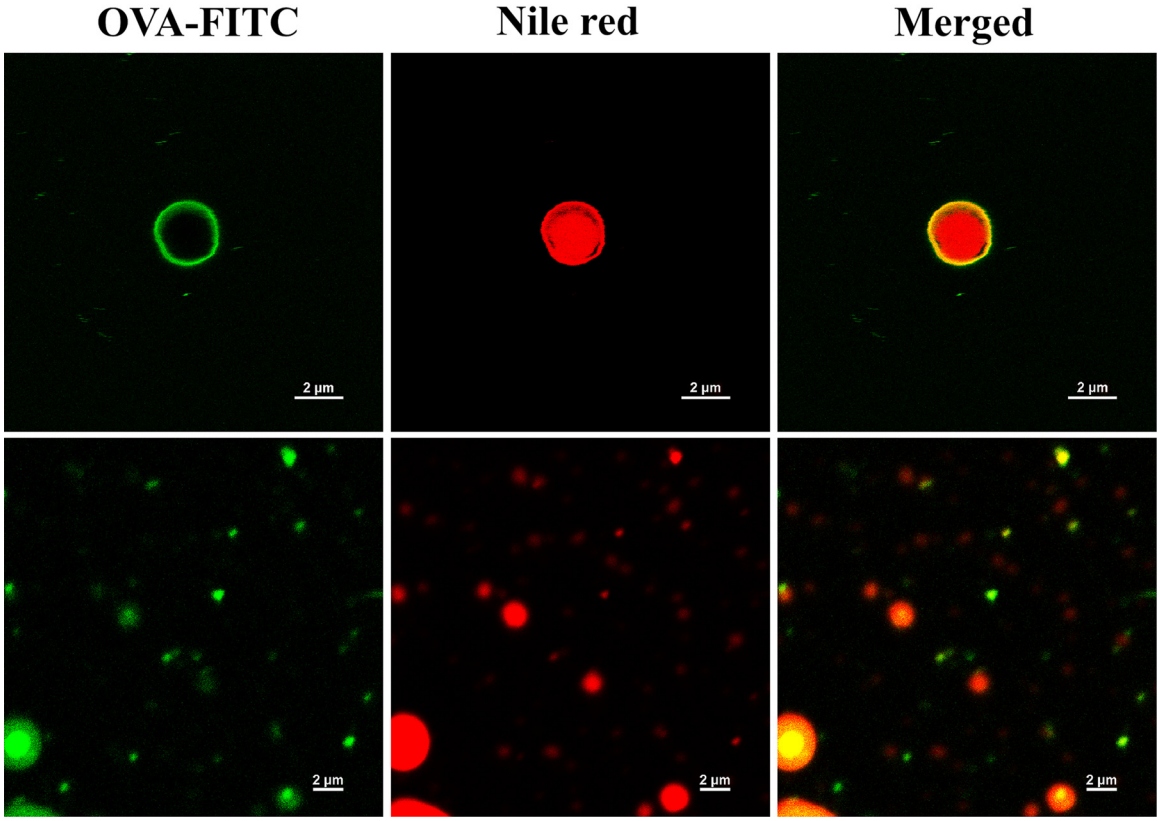

Figure 2. Confocal laser scanning microscopy antigen loaded PPAS. Model antigen OVA-FITC was in green, oil phase squalene of PPAS is red.

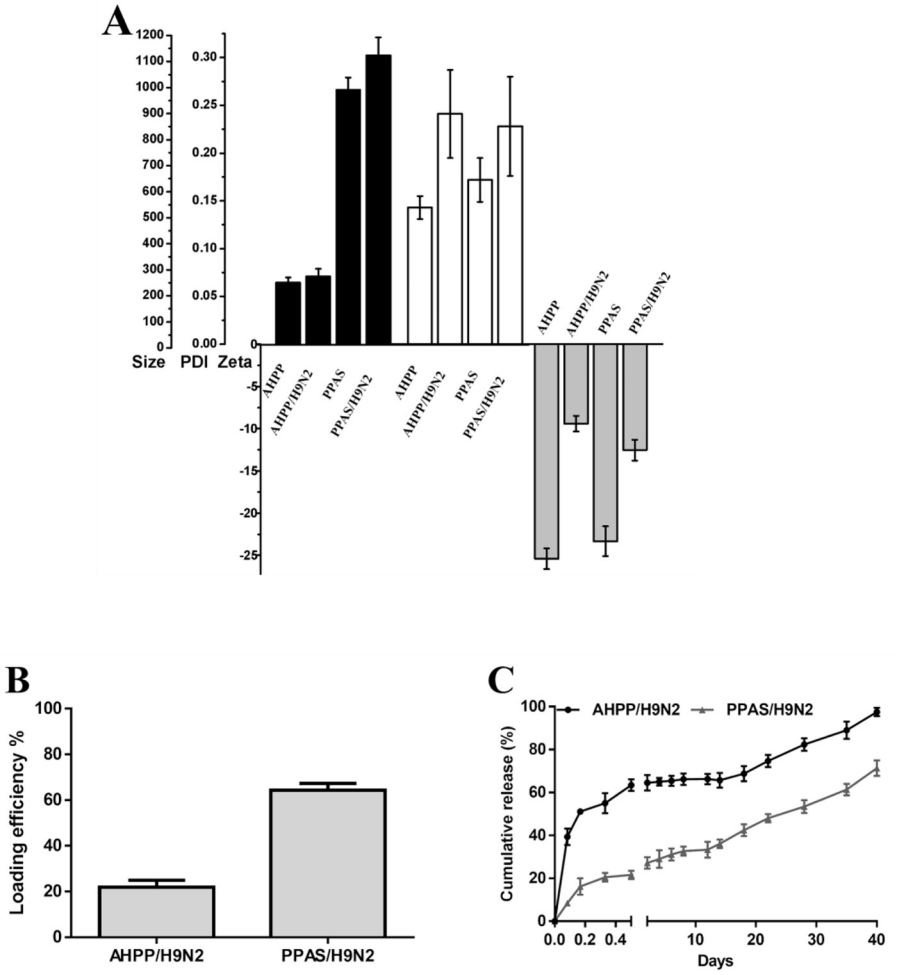

Figure 3. (A) Change of size, PDI, and Zeta potential of pre- and post-H9N2 loaded nanoparticle. (B) H9N2 antigen adsorption morphology of AHPP and PPAS. (C) In vitro release of H9N2 from AHPP/H9N2 and PPAS/H9N2. Data are expressed as the mean ± SEM (n = 4).

HI Antibody Titers

As shown in Figure 4A, AHPP/H9N2, PPAS/H9N2, and positive control Alum/H9N2 could induce long-lasting and significantly higher HI titers from d 7 to d 35 than H9N2 group ($P < 0.05$). Among them, PPAS/H9N2 induced the strongest HI titer at d 21 and 35 in all groups, but there was no significant difference with AHPP/H9N2 and PPAS/H9N2 groups ($P > 0.05$).

The immune organ index is an important index reflecting the immune status of the body. As shown in Figures 4B and 4C, PPAS/H9N2 and Alum/H9N2 exhibited high thymus, spleen, and bursa of fabricius organ index compared to other groups. The thymus index of PPAS/H9N2 on d 21 and 35 was significantly higher than that of H9N2 and PBS groups ($P > 0.05$).

Differentiation of $CD4^+$ and $CD8a^+$ T-Cells

Chicken was intramuscular injected with different formulations, and then the differentiation of $CD4^+$ and $CD8a^+$ T-cells in the splenic lymphocyte were detected at the d 21 after the final immunization. As shown in Figures 7A and 7B, chicken in AHPP/H9N2 and PPAS/H9N2 group displayed significantly higher quantity of $CD3e^+$ $CD4^+$ and $CD3e^+CD8^+$ T cells expression than all other groups ($P < 0.05$). The differentiation levels of $CD3e^+$ $CD4^+$ and $CD3e^+CD8^+$ T cells in PPAS/H9N2 groups were higher than those in AHPP/H9N2 groups ($P > 0.05$). The above results indicate that PPAS/H9N2 could improve the differentiation of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells.

Expression of Cytokines mRNA

The PPAS/H9N2 formulations induced highest levels of IL-4 and IL-6 (Th2-type cytokines), and IFN-γ and IL-2 (Th1-type cytokines) among all of the groups. The Th1-type cytokines IFN-γ and IL-2 levels induced by PPAS/H9N2 group was significantly higher than that in AHPP/H9N2 and Alum/H9N2 groups. The Th2-type cytokines IL-4 and IL-6 levels induced by PPAS/H9N2 group was significantly higher than that in Alum/H9N2 groups, but there was no significantly significant different with PPAS/H9N2 group (Figures 6A and 6B). These results indicated that PPAS/H9N2 formulations could induce a strong mixture secretion of Th1 and Th2 mediated responses.

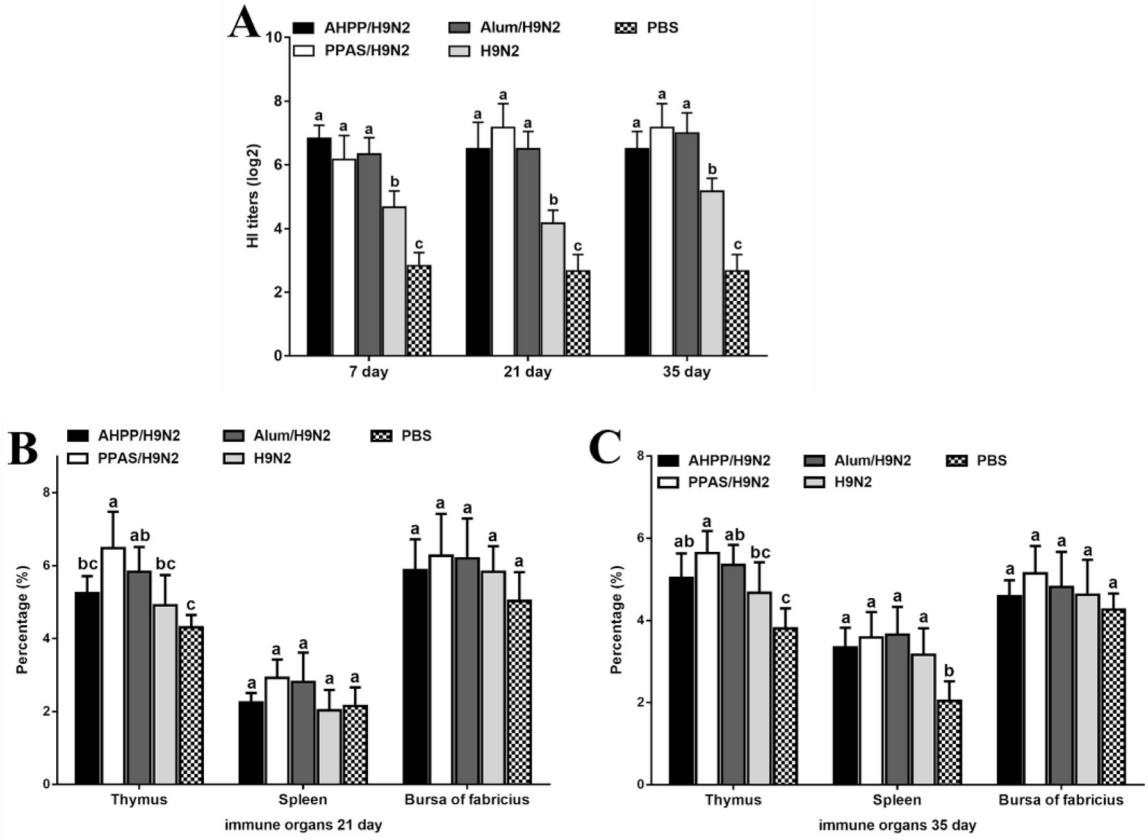

Figure 4. (A) The expression of HI titers. (B, C) Immune organ index at d 21 and 35. [a–c] Bars with different superscripts differed significantly ($P < 0.05$), n = 5.

Histology Analysis

The thymus, spleen, and bursa of fabricius were collected on d 35 after the second immunization. The collected organs were performed by HE staining (Figure 7). The results showed that there were no obvious toxicities or inflammatory infiltrates in all the experimental groups, which indicated the safety of all the H9N2-loaded nanoparticles.

DISCUSSION

H9N2 is the most widely distributed influenza virus in the world had caused serious economic losses in the poultry industry worldwide (Lee et al., 2008; Uddin et al., 2015; Yitbarek et al., 2019). Vaccination remains the most effective and economically prudent strategy to counter the threat posed by the H9N2 avian influenza virus (Nicholson et al., 2010; Golchin et al., 2017; Su et al., 2020). H9N2 influenza vaccine combined with commercial adjuvants such as alum adjuvant or oil emulsion adjuvant-induced strong humoral immune responses, but with a poor cellular immune response (Atmar et al., 2006; Riaz et al., 2017). The cellular immune response is crucial for the host defence system against infection (Xie et al., 2012; Marjolein et al., 2020). Moreover, it has been reported that in addition to humoral immunity, effective cellular responses also play a crucial role in protection against H9N2 infection (Jafari et al., 2017). Therefore, preparing a vaccine adjuvant that can induce an effective mixture of cellular and humoral immune responses is critical to defence against H9N2 influenza. PLGA is a biodegradable material that has been widely investigated due to its safety, antigen slow-release ability, and good adjuvants activity. (Dhakal et al., 2017; Mir et al., 2017). AHP has been demonstrated that possesses excellent immune enhancement, and widely used as immune-stimulator (Wusiman et al., 2019a). In our previous research, AHP encapsulated PLGA nanoparticles have been used as an effective nano-adjuvant to improve the immune effect of antigens (Wusiman et al., 2019b). However, the antigen adsorption rate of AHPP is low. During the preparation of AHPP, the antigen will be indirectly in contact with organic solvents, inevitably exposing antigens to harsh conditions.

In this study, PLGA nanoparticles based PPAS were prepared by assembling AHPP with oil-phase squalene via phacoemulsification. From the SEM and Cryo-SEM images, it was clear that AHPP has a spherical shape and a smooth surface was about 200 nm in size. PPAS also has a spherical shape but with rough strawberry-like surfaces was about 1 μm in size. A large number of H9N2 vaccines can successfully adsorb on the surface of the PPAS. The antigen adsorption morphology, changes of particle size, and high antigen loading efficiency verified this effect. As shown in Figures 2 and 3A, a large among of antigens will be adsorbed on the strawberry-

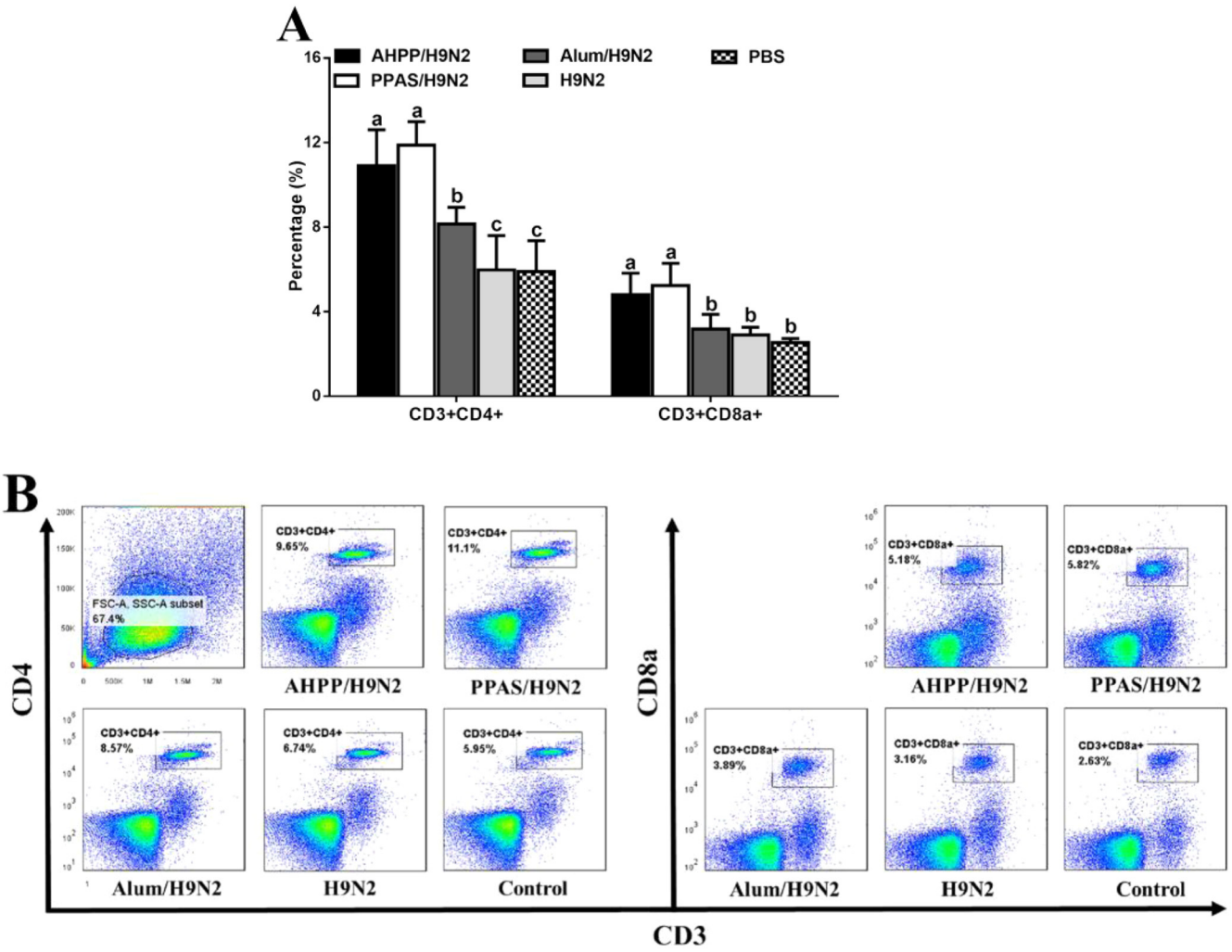

Figure 5. Differentiation of $CD4^+$ and $CD8a^+$ T cell in spleen. (A) Expression of $CD4^+$ and $CD8a^+$ T cell. (B) Differentiation of $CD4^+$ and $CD8a^+$ T cell in splenic T lymphocytes. $^{a-c}$ Bars with different superscripts differed significantly ($P < 0.05$), n = 4.

like surface of the PPAS after simple mixing so that the particle size increases by 100 nm. The adsorption capacity of AHPP is weak, so the particle size did not change significantly. The antigen loading rate of AHPP/H9N2 and PPAS/H9N2 were 24.6 ± 1.91% and 64.7 ± 3.34% (Figure 3B); this result was consistent with the results reported above.

Vaccines are the best defence against infectious diseases (Chrzastek et al., 2018; Shin-Hee, 2018). H9N2 infection is usually defended by H9N2 vaccine, but due to poor immunogenicity of vaccine antigen, adjuvants need to be added to enhance the immune effect of the H9N2 vaccine (Kilany et al., 2016; Riaz et al., 2017). However, commercial adjuvants such as aluminum adjuvants and oil adjuvants are limited in their clinical application due to their defects, such as inability to induce effective cellular immune responses or large toxic and side effects (Kilany et al., 2016; Irshad et al., 2018). It is necessary to develop safe and effective adjuvants to induce strong humoral and cellular immune responses. In this study, the adjuvant effects of the AHPP and PPAS for the H9N2 vaccine were evaluated. It was found that PPAS/H9N2 induced long-lasting and high HI titer comparable to the other groups and promoted a high immune organ index (Figures 4A−4C). Furthermore, in all treatment groups, PPAS/H9N2 significantly increased the differentiation of $CD3e^+$ $CD4^+$ and $CD3e^+CD8^+$ T cells in the spleen compared to Alum/H9N2 group (Figures 5A and 5B). Alum adjuvant is known to induce strong Th-2 polarized humoral immune responses (Hawken and Troy, 2012; Patel et al., 2006). As such, these data suggested that the PPAS/H9N2 not only induced a strong Th-2 type immune response but also mediates an effective Th-1 type immune response. As shown in Figures 6A and 6B, PPAS/H9N2 promoted the mRNA expression of IFN-γ, IL-2, IL-4, and IL-6 cytokines, further suggesting that PPAS/H9N2 induced a mixed Th1 and Th2 immune response. Histology analysis of immune organs from PPAS immunized chickens indicated the safety of the PPAS/H9N2 formulations. Therefore, these results show that AHPP based assembled particles-oil adjuvants PPAS nanoparticles provide an excellent alternative for adjuvant development of the H9N2 vaccine.

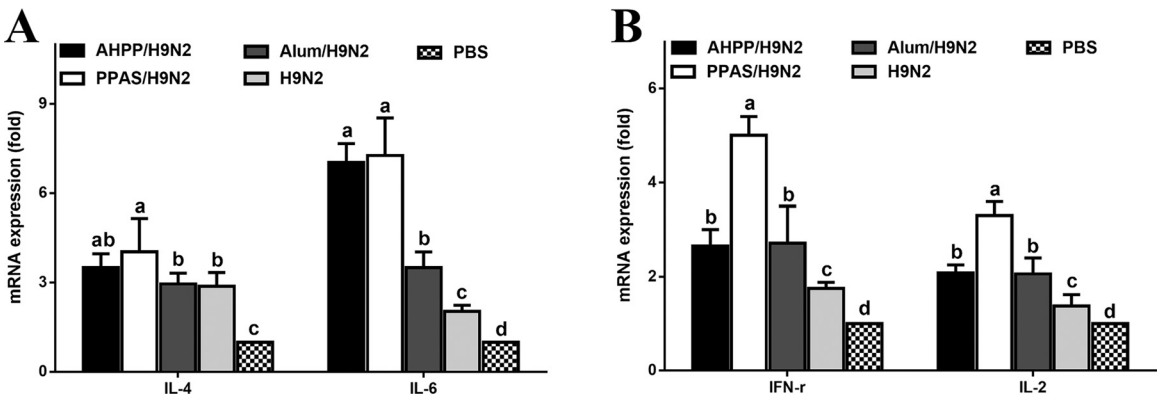

Figure 6. Relative mRNA expression levels of (A) IL-4 and IL-6, (B) IFN-γ and IL-2 cytokines in chicken peripheral blood lymphocytes. [a-d] Bars in the histogram with different letters were significantly different ($P < 0.05$). n = 4.

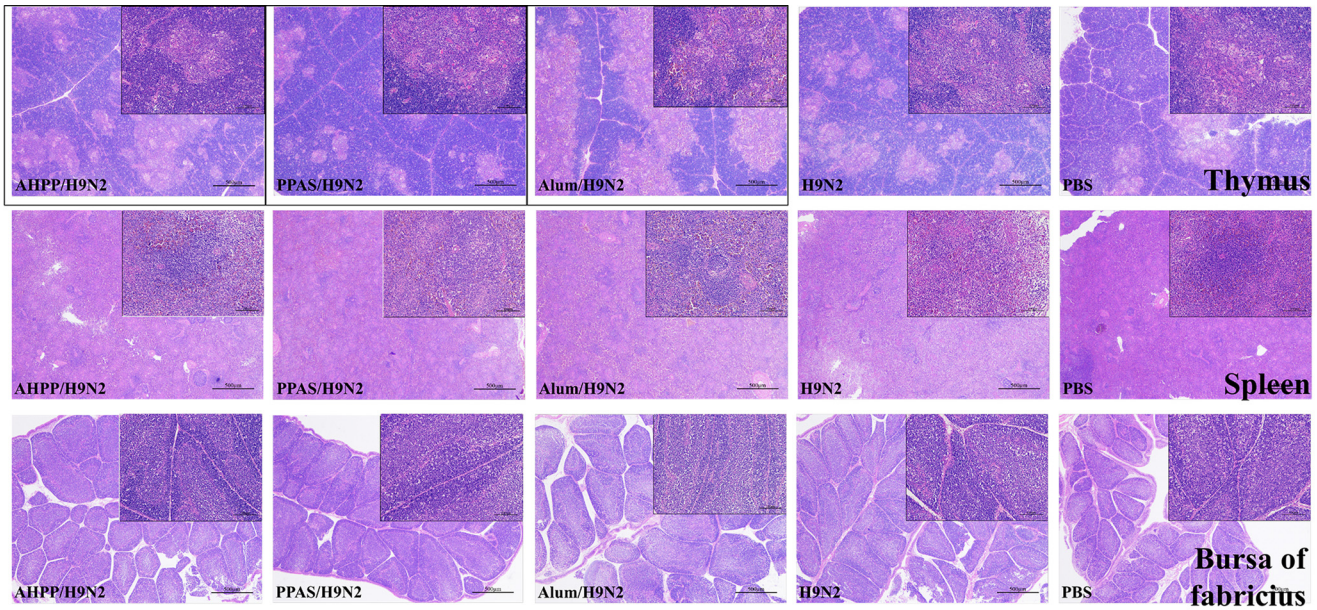

Figure 7. Histological analysis of thymus, spleen, and Bursa of Fabricius in chickens (200 ×, HE, scale bar was 100 μm).

CONCLUSIONS

In summary, to our knowledge, this is the first study to synthesize, characterization and in vivo evaluation of the PLGA nanoparticles and PLGA nanoparticles assembled pickering emulsion. Among the 2 types of nanoparticles, PPAS could load a large amount of H9N2 antigen by simple mixing showed a good antigen sustained-release effect. PPAS/H9N2 immunized chicken can induce high HI antibody titer, immune organ index, a mixed $CD4^+$ and $CD8a^+$ T cell differentiation, and Th1 and Th2-type cytokines mRNA expression. The acquired data on PPAS/H9N2 formulation is potentially served as a novel and effective vaccine adjuvant in chicken to induce specific immune responses against infections and diseases.

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DISCLOSURES

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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