Vaccine 37 (2019) 4291–4301

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

An amphiphilic invertible polymer as a delivery vehicle for a M2e-HA2-HA1 peptide vaccine against an Influenza A virus in pigs

Gagandeep Singh [a], Oksana Zholobko [b], Angela Pillatzki [c], Brett Webb [d], Eric Nelson [c], Andriy Voronov [b], Sheela Ramamoorthy [a,*]

[a] Department of Microbiological Sciences, N. Dakota State University, Fargo, ND, United States
[b] Department of Coatings and Polymeric Materials, N. Dakota State University, Fargo, ND, United States
[c] Animal Disease Research and Diagnostic Laboratory, S. Dakota State University, Brookings, SD, United States
[d] Veterinary Diagnostic Laboratory, N. Dakota State University, Fargo, ND, United States

ARTICLE INFO

Article history:
Received 20 November 2018
Received in revised form 11 June 2019
Accepted 17 June 2019
Available online 21 June 2019

Keywords:
M2e
HA2
HA1
Influenza
Vaccine
Peptide
Amphiphilic invertible polymer
Micellar assemblies
H1N1
Swine influenza virus

ABSTRACT

Influenza A viruses (IAVs) are a group of genetically diverse and economically important zoonotic pathogens. Despite decades of research, effective and broadly protective vaccines are yet to be developed. Recent breakthroughs in epitope-based immunization for influenza viruses identify certain conserved regions of the HA2 and M2e proteins as capable of inducing broad protection against multiple influenza strains. The M2e and HA2 peptides have been evaluated in mice but not as a combination in pigs, which play an important role in the transmission and evolution of IAV. Peptides are inherently weak immunogens; and effective delivery of peptide antigens is challenging. To enhance the delivery and immunogenicity of peptide-based vaccines, the conserved M2e and HA2 and a strain-specific HA1 epitope of Influenza A (H1N1) pdm09 were expressed as a chain in a bacterial expression system and entrapped in a novel amphiphilic invertible polymer made from polyethyelene glycol (PEG, molecular weight 600 g/mol) and polytetrahydrofuran (PTHF, molecular weight 650 g/mol), $PEG_{600}PTHF_{650}$. Piglets vaccinated with polymeric peptide vaccine mounted significantly stronger antibody responses against the peptide construct when compared to piglets immunized with the multi-epitope peptide alone. When vaccinated pigs were challenged with Influenza A (H1N1) pdm09, viral shedding in nasal secretions and lung lesion scores were significantly reduced when compared to the unvaccinated controls and pigs vaccinated with the peptide alone at six days post-challenge. Thus, the combination of the $PEG_{600}PTHF_{650}$ polymer and trimeric peptide construct enhanced delivery of the peptide antigen, acted as an adjuvant in stimulating strong antibody responses, reduced the effects of viral infection in vaccinated pigs.

1. Introduction

Influenza A viruses (IAV) of the *Orthomyxoviridae* family are important zoonotic pathogens. Genetic and antigenic variation associated with IAV renders the successful development of broadly-protective human and swine vaccines against IAV a long-standing challenge. Pigs serve as "mixing vessels" for human and avian influenza viruses, supporting the emergence of new influenza virus strains [1,2]. Thus, vaccines which provide effective and broad protection against several strains of influenza virus in pigs would be very valuable for animal and public health.

Recently, vaccines containing certain conserved antigenic epitopes of influenza viruses were shown to elicit broad protection against a number of genetically diverse strains in mouse models [3–12]. The extracellular N-terminal domain of the M2 protein (M2e) is a 23 amino acid peptide which is highly conserved in all influenza A viruses [13]. M2e-based peptide vaccines were shown to provide heterogenetic immunity against IAV in mice, but were not as effective in swine models [10,14,15]. Similarly, the fusion peptide located in the HA2 protein is highly conserved among the different influenza virus strains [13,16,17] and provided broad protection in mice, but has not been tested in swine [18,19]. A third epitope, ATGLRNIPSIQSRLE, located in the HA1 region, was selected for its protective potential against H1 subtype viruses [20]. A combination of the HA2 and M2e peptides has not been tested in pigs [3–12]. In this study, we test the hypothesis that a polypeptide encoding a combination of the M2e and HA2 conserved epitopes with one type specific epitope would be effective in preventing IAV infection in pigs [13].

* Corresponding author.
E-mail address: sheela.ramamoorthy@ndsu.edu (S. Ramamoorthy).

https://doi.org/10.1016/j.vaccine.2019.06.030
0264-410X/

While highly specific, small peptides are weak immunogens and vulnerable to degradation [21], necessitating the development of improved systems the delivery of peptide vaccine. Polymers are well-established as substances that can enhance vaccine delivery, reduce dosage, and act as adjuvants, to broaden vaccine-induced immune protection. In addition, self-assembled polymeric architectures can increase the duration of immunity due to slow and sustained release of the antigen over time [22–24]. Amphiphilic polymers which enable customizing of peptide antigen delivery by varying the macromolecular structure and also act as adjuvants provide significant value to the development of peptide vaccines [25]. We have previously synthesized a library of amphiphilic invertible polymers (AIPs) which self-assemble into polymeric micelles as AIP concentration increases, both in polar and nonpolar solvents, and can rapidly switch their conformation in response to changes in the environmental polarity, thus facilitating the micellar inversion [26,27]. The AIP conformational inversion is a promising tool for rapid and controlled self-assembly in applications that require simultaneous utility in polar and nonpolar media, e.g., in drug delivery systems. In our previous studies, the incorporation of two different peptides into micellar assemblies of AIP, made from polyethylene glycol (PEG, molecular weight 600 g/mol) and polytetrahydrofuran (PTHF, molecular weight 650 g/mol), $PEG_{600}PTHF_{650}$, were characterized and described in detail [28]. The micellar inversion of this AIP loaded with peptides was also demonstrated [29].

In this study, the three selected influenza A virus epitopes described above (conserved M2e, HA2, and type-specific HA1) were expressed as a chain in a bacterial expression system and incorporated into the $PEG_{600}PTHF_{650}$ micellar assemblies. The efficacy of the AIP micellar assemblies as a peptide antigen delivery system was evaluated in vitro and in vivo in using a swine model. The data presented below demonstrates that the $PEG_{600}PTHF_{650}$ micellar assemblies are effective in delivering the peptide cargo to cells, and act as an adjuvant in stimulating strong antibody responses against the delivered antigen in vaccinated pigs.

2. Materials and methods

All experiments described below were carried out in compliance with the Institutional Biosafety Committee (IBC) and Institutional Animal Care and Use Committee (IACUC) regulations of North Dakota State University (NDSU) and South Dakota State University (SDSU).

2.1. Cells and viruses

To prepare the virus stock culture for both the challenge of vaccinated pigs and for the hemagglutination inhibition (HI) assay, Influenza A/California/04/09 H1N1 virus was cultured using Madin-Darby Canine Kidney (MDCK) cells as previously described [30]. After 48 h of incubation, virus particles were harvested by three freeze-thaw cycles, followed by clarification at 10,000*g* for 10 min at 4 °C to remove cellular debris. The titer was assessed using the tissue culture infectious dose 50% [$TCID_{50}$] assay and the Reed–Muench formula [31].

2.2. Preparation of the peptide antigen

The coding sequences for the conserved peptides which were previously identified as broadly protective (343 RGLFGAIAGFIEGGW 357 and 2GSSLLTEVE TPTRSEWECRCSD 21), containing the fusion peptide of the HA2 protein and the ectodomain of the M2 protein respectively, were incorporated in the construct. In addition, a strain-specific, linear HA1 epitope (321 ATGLRNIPSIQSRLE 333), also previously demonstrated to be protective in mice and located in the C terminus of the HA1 protein adjacent to the inter-subunit region near the protease cleavage site [32–34], was also expressed. The coding sequences for the peptides were commercially synthesized (Integrated DNA Technologies, USA) as a chain with glycine-serine linkers and BamHI and NcoI restriction sites on the ends. All peptide sequences were generated from Influenza A/California/04/09 H1N1) [35], The synthesized DNA with encoding the sequence MGSSHHHHHHSSGLV PRGSHM ASMTGGQQMGRGSSLLTEVETPTRSEWECRC SDSSGSGSGSR GLFGAI AGFIEGGWGGGGSGGGGSATGLRNIPSIQSRLEHHHHHH was inserted into the pET28a (+) (MilliporeSigma, USA) bacterial protein expression vector. The vaccine antigen was prepared by bacterially expressing the peptide encoding three IAV epitopes. The expressed recombinant peptide was purified by using Ni-NTA affinity chromatography under native conditions and characterized by a western blot using an anti-M2e peptide-specific monoclonal antibody. The purified M2e-HA1-HA2 peptide was dissolved in dimethyl sulfoxide (DMSO) and stored in aliquots at −80 °C until further use.

2.3. Amphiphilic invertible polymer (AIP) synthesis

The AIP, $PEG_{600}PTHF_{650}$, was synthesized as previously reported from PEG (molecular weight 600 g/mol) and PTHF (molecular weight 650 g/mol) using a polycondensation reaction [27,36]. Chemical composition of $PEG_{600}PTHF_{650}$ was confirmed by FTIR and ^1H NMR spectroscopy. Average molecular weights and the corresponding polydispersity index of the AIP was measured using gel permeation chromatography (GPC) (data not shown).

2.4. ***Cellular cytotoxicity of $PEG_{600}PTHF_{650}$*** *micellar assemblies*

To ensure the $PEG_{600}PTHF_{650}$ is not toxic to cells, cytotoxicity of polymer micellar assemblies was assessed in vitro using MTT (3-(4,5-dimethylthiazol-2-yl) Tr-2,5-diphenyltetrazolium- bromide) (Sigma-Aldrich, USA) [37]. Micellar assemblies were prepared using different concentrations of $PEG_{600}PTHF_{650}$ (0.05, 0.2, 0.5, 0.75 and 1.0 w/v%) by the thin film technique using DMSO as solvent [28]. One hundred μl/well of each prepared solution was added into 96 well cell culture plates (VWR, USA) containing monolayers of Vero cells and incubated for 8 h at 37 °C in a CO_2 incubator. After incubation, the solution from the wells was removed and the wells were washed three times with Hank's balanced salt solution (HBSS). The MTT was dissolved to 0.5 mg/ml in sterile PBS, and 100 μl was added to each well. Plates were incubated in a CO_2 incubator for 4 h to reduce MTT into formazan. The supernatant from each well was carefully removed by aspiration without disturbing the cells. To solubilize the intracellular formazan, 100 μl of DMSO was added to each well, mixed well by vigorous pipetting, and incubated for 5 min. Plates were read at 570 nm in microplate reader.

2.5. Interaction between $PEG_{600}PTHF_{650}$ micellar assemblies and M2e-HA1-HA2 peptide

To demonstrate the interaction between $PEG_{600}PTHF_{650}$ micellar assemblies and M2e-HA1-HA2 peptide, ^1H NMR spectra were recorded on an AVANCE III HDTM 400 high-performance digital NMR spectrometer at 400 MHz and 22.5 °C. Proton spectra were collected for 0.5 w/v% PEG_{600}-$PTHF_{650}$ or polymer-peptide micellar assemblies containing 0.005 w/v% peptide added to 0.5 w/v% $PEG_{600}PTHF_{650}$ formed in deuterated water by thin film technique [27]. The spectra were obtained for polymer samples with or without the peptide and referenced to a 3-(Trimethylsilyl)

propionic-2,2,3,3-d4 acid sodium salt (TMSP) signal as an internal standard (Fig. 1).

2.6. Relative antigen loading capacity

The peptide loading capacity of the $PEG_{600}PTHF_{650}$ micellar assemblies was assessed by a whole cell enzyme-linked immunosorbent assay (ELISA), capable of detecting intracellular peptide. Polymer-peptide micellar assemblies were prepared using different concentrations of $PEG_{600}PTHF_{650}$ (0.05, 0.2, 0.5, 0.75, and 1.0 w/v%) and a constant M2e-HA1-HA2 peptide concentration of 0.5 μg/μl. One hundred μl/well of prepared assemblies were added into 96 well cell culture plates (VWR, USA) containing a monolayer of Vero cells, and incubated for 8 h at 37 °C in a CO_2 incubator. The peptide alone or wells with no treatment served as controls. After 8 h incubation, the solution from wells was removed, and wells were washed three times with phosphate buffered saline with tween (PBST). To each well, 100 μl of anti-M2e monoclonal primary antibody (provided by Dr. Elieen Thacker, U.S National Poultry Research Center, Athens, GA) diluted 1:100 in blocking buffer (PBST + 2% Bovine serum albumin, BSA) was added and incubated for 1 h at 37 °C with 5% CO_2 followed by washing. 100 μl of HRPO conjugated anti-mouse IgG secondary antibody (KPL, USA) was added and incubated for 1 h at 37 °C with 5% CO_2. After washing, the reaction was developed with 3,3′,5,5′-tetramethylbenzidine substrate (TMB) (KPL, USA) and stopped after 15 min with 1 M Hydrochloric acid. The optical density values were read at 450 nm using a microplate reader (BioTek Instruments, Winooski, VT) (Fig. 2).

2.7. In-vitro peptide delivery by Immuno-Fluorescence assay (IFA)

The effectiveness of the $PEG_{600}PTHF_{650}$ micelles in delivering the M2e-HA2-HA1 peptide into the cells was observed by an immunofluorescence (IFA) assay. Polymer-peptide assemblies were prepared using 1% w/v of $PEG_{600}PTHF_{650}$ and 0.5 μg/μl

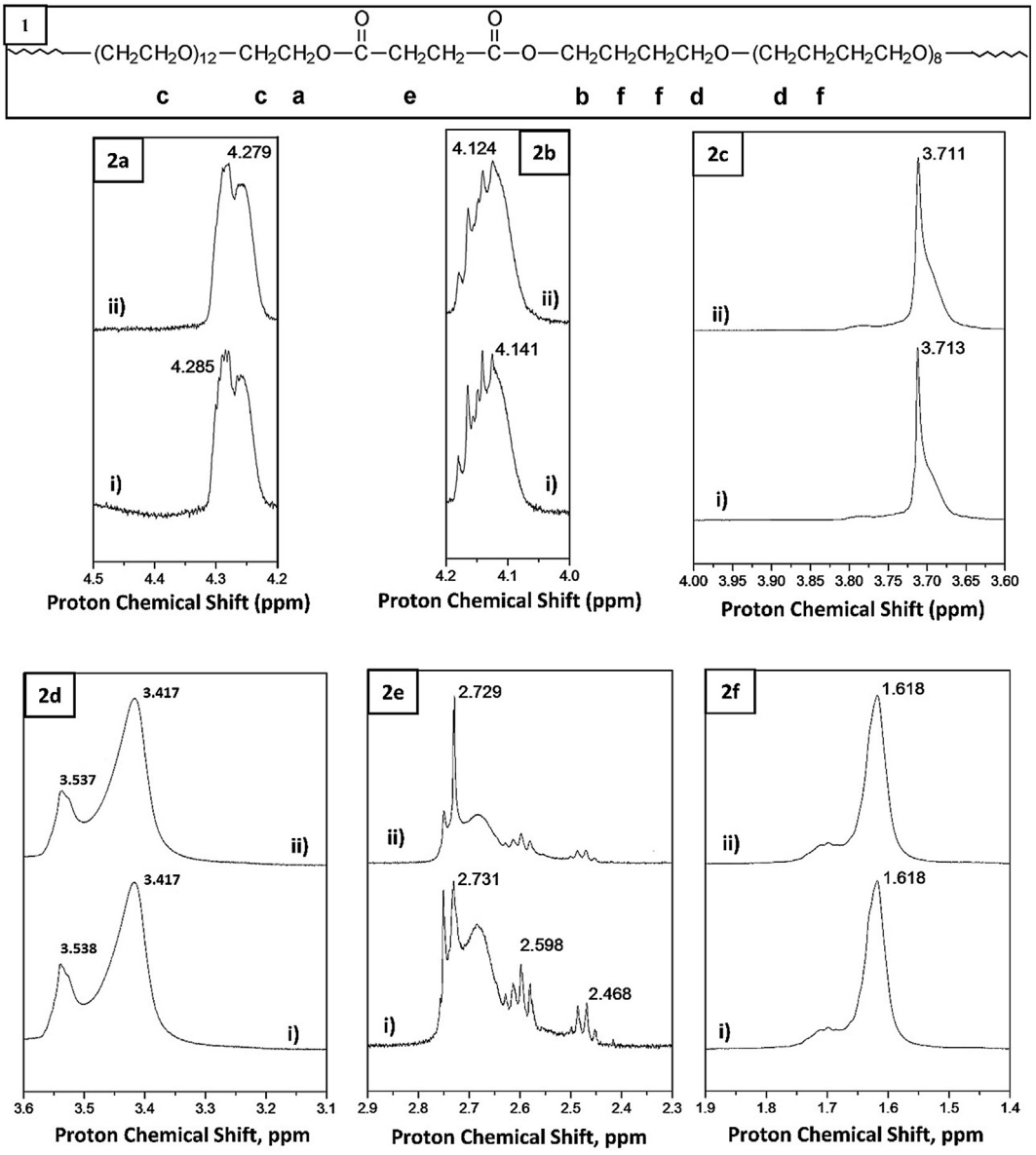

Fig. 1. ^1H NMR spectra of the $PEG_{600}PTHF_{650}$ solution (0.5 w/v%) in D_2O. Panel 1- The chemical structure of $PEG_{600}PTHF_{650}$ with protons labeled a–f. Panel 2a through 2f correspond to the peaks representing the protons a–f depicted in panel 1 respectively. X-axis – proton chemical shift measured as ppm (parts per million). (i) Spectrum of the 0.5 w/v% $PEG_{600}PTHF_{650}$ solution alone (ii) Shift in spectrum after the addition of M2e-HA1-HA2 peptide to the 0.5 w/v% $PEG_{600}PTHF_{650}$ solution.

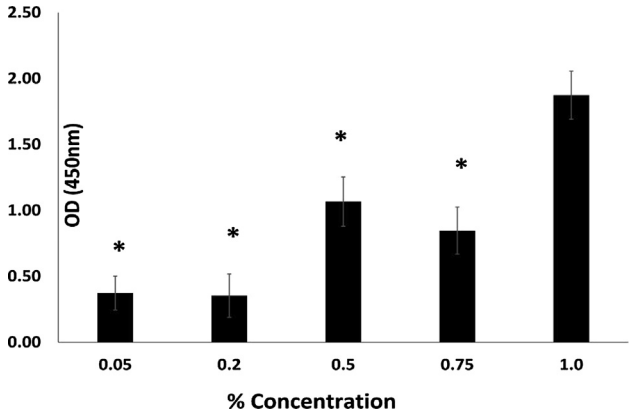

Fig. 2. Antigen loading capacity of $PEG_{600}PTHF_{650}$ micellar assemblies. Intracellular delivery of peptide antigen as measured by an antigen detection ELISA using an M2e peptide-specific monoclonal antibody. Y-Axis: optical density (OD) at 450 nm, Y axis – w/v% concentration of $PEG_{600}PTHF_{650}$. Vero cells monolayers incubated with micellar assemblies prepared with 0.05, 0.2, 0.5, 0.75, and 1.0 w/v% of PEG_{600}-$PTHF_{650}$ and a constant concentration of 0.5 μg/μl of the M2e-HA1-HA2 peptide for 8 h. Intracellular delivery of antigen was detected by ELISA using a M2e peptide-specific monoclonal detecting antibody after 24 h. * – significantly different from micellar assemblies prepared from 1.0 w/v% polymer ($P < 0.05$, Student's T test). No signal was detected in cells treated with the peptide or $PEG_{600}PTHF_{650}$ controls alone (data not shown).

M2e-HA2-HA1. The prepared solution was added at 100 μl/well into 8-well Nunc® Lab-Tek™ chamber slides containing a Vero cell monolayer and incubated for 1, 2, 4, and 8 h at 37 °C with 5% CO_2. Wells with no treatment or wells incubated with peptide alone were used as controls. After incubation, the solution was aspirated from the wells and wells were washed three times with HBSS. To detect intracellular antigen, cells were fixed using chilled acetone: methanol (1:1). Following overnight fixation, the chamber slides were washed three times using PBST and 100 μl of 1:100 anti-M2e monoclonal antibody diluted in blocking buffer was added to each well, incubated at 37 °C for 1 h and washed with PBST. 100 μl of 1:500 anti-mouse IgG fluorescein-conjugated secondary antibody (KPL, USA) was added to each well and incubated at 37 °C for 1 h. Washed slides were mounted with 50% glycerol, followed by fluorescent microscopic examination (Fig. 3).

2.8. Vaccine formulation

Twenty-four, 3-week old, IAV-S negative piglets of both sexes were obtained from the swine research facilities as North Dakota State University. This closed, high-health herd is negative for all major swine pathogens including influenza and animals are not vaccinated against influenza viruses. Piglets were assigned to four groups as follows: Group I – Unvaccinated control (N = 7), Group II –$PEG_{600}PTHF_{650}$/M2e-HA1-HA2 peptide micelles (IAV-S-VAC)

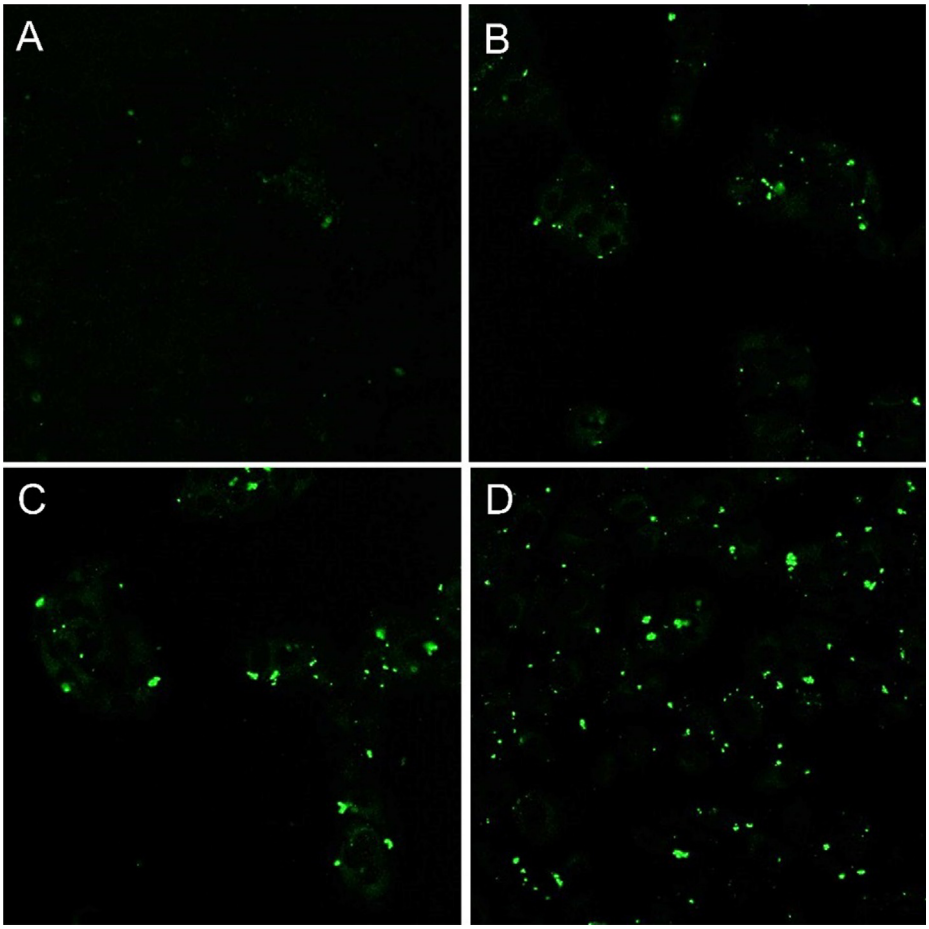

Fig. 3. Intracellular delivery of M2e-HA1-HA2: Vero cell monolayers were incubated with micellar assemblies prepared with 1.0 w/v% of $PEG_{600}PTHF_{650}$ and 0.5 μg/μl of M2e-HA1-HA2 peptide for 1 h (A), 2 h (B), 4 h (C) and 8 h (D) and assessed by IFA using a M2e peptide-specific monoclonal antibody. Increasing green fluorescence is indicative of increasing accumulation of intracellular peptide over time. No signal was detected in cells treated with the peptide or $PEG_{600}PTHF_{650}$ controls alone (data not shown). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(N = 7), Group III – M2e-HA1-HA2 peptide only (N = 5) or Group IV-$PEG_{600}PTHF_{650}$ polymer assemblies only (N = 5). For each vaccine dose, 0.8 ml of M2e-HA1-HA2 peptide (1 mg/ml) dissolved in DMSO was added to a 30 ml Pyrex glass vial containing 40 mg of $PEG_{600}PTHF_{650}$ and mixed well by vortexing. A thin film was prepared and subsequently hydrated with 4 ml of DMEM to form $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 micellar assemblies. For each animal in the M2e-HA1-HA2 peptide control group, a thin film was obtained from 0.8 ml of M2e-HA1-HA2 peptide (1 mg/ml) dissolved in DMSO and hydrated with 4 ml of DMEM to prepare the M2e-HA1-HA2 peptide solution. Similarly, for each animal in the $PEG_{600}PTHF_{650}$ control group, a thin film was obtained from 40 mg of $PEG_{600}PTHF_{650}$ dissolved in DMSO hydrated with 4 ml of DMEM to prepare 1w/v% of the $PEG_{600}PTHF_{650}$ solution. Therefore, the effective vaccine dose for each piglet was 0.8 mg (0.2 μg/μl) of M2e-HA1-HA2 peptide antigen and 1 w/v% of PEG_{600}-$PTHF_{650}$ polymer per dose.

2.9. Swine immunization and challenge

At the day of vaccination (DPV 0), piglets in each group were treated with 4 ml of innocula, 2ml delivered intranasally and 2 ml subcutaneously as described above. At the second and third week post-vaccination (DPV 14 and DPV 20, respectively) piglets were boosted with the same doses and routes. At DPV 35 (0 day post-challenge or DPC 0), two pigs from group I and group II were sacrificed prior to challenge with Influenza A (H1N1) pdm09 virus, to assess vaccine safety. All remaining pigs were challenged intranasally with 4 ml of $10^{5.5}$ $TCID_{50}$/ml of Influenza A/California/04/09 H1N1 culture. All piglets were euthanized by barbiturate overdose at 41 DPV (DPC 6) for necropsy. Pathology procedures were carried out as described below. Serum was collected from all piglets on DPV 0, 14, 20, 35 and 41 to detect antibodies to the peptide by ELISA. Nasal swabs were collected from all piglets at DPV 35, 38 and 41 (or DPC 0, 3 and 6) and tested by qPCR for shedding of the challenge virus.

2.10. Clinical observation and pathological examination

Piglets were observed every day post-challenge for clinical signs of IAV-S including fever, nasal discharge, coughing, anorexia, and lethargy. Weight and temperatures were measured every day post-challenge.

Pathological evaluation and scoring was carried out in a blinded fashion by a board-certified veterinary pathologist. Heart, liver, spleen, kidney and lymph node tissues were collected from two pigs each euthanized prior to challenge from the vaccine group and unvaccinated control group to assess vaccine safety [38]. Lung sections were prepared from the right and left cranial, medial and caudal lobes and accessory lobes. Hematoxylin and eosin-stained tissue sections were observed for microscopic changes indicating viral infection. In addition, the lung sections were stained with the anti-M2e monoclonal antibody to determine localization of the M2e-HA1-HA2 peptide by immunohistochemistry (IHC).

For the remaining animals sacrificed on the 6th day after challenge, protection against the development of gross lesions due to virulent viral challenge was assessed as the percentage of the tissue affected in each of the six lung regions listed above. The total percentage of lungs affected for the 5 pigs/group is shown in Table 1. Similarly, microscopic lesions were assessed using hematoxylin and eosin stained sections, as previously described, with some modifications [39,40]. Briefly, bronchial/bronchiolar epithelial changes, and/or bronchitis and bronchiolitis were assessed as a percentage value for each of the six lung sections examined using the following scoring matrix: 25% airways affected = 1, 26–50% airways affected = 2, 51–75% airways affected = 3, 76–100% airways affected = 4. Interstitial pneumonia (IP) was scored as 0 = none, 1 = mild, focal to multifocal IP, 2 = moderate, locally extensive to multifocal IP, 3 = moderate, multifocal to coalescing IP, 4 = severe, coalescing to diffuse. Peribronchiolar lymphocytic cuffing was scored as 0 = none, 1 = minimal, loosely formed, 2 = mild, loosely formed, 3 = moderate, well formed, 4 = severe, thick, well-formed cuffs. Total values were calculated as a sum for the five pigs per group (Table 1).

Lung sections were stained with an IAV-S specific monoclonal antibody (ATCC Hb65) for IHC and scored as weak = 1, moderate = 2, strong = 3. The sum of the number of sections positive for antigen and the scores for each group is listed in Table 1. Consolidated total lesion scores were calculated as the sum of the gross, microscopic and IHC scores per group. The Mann-Whitney *U* test was applied to determine whether there were significant differences between groups at $p \leq 0.05$.

2.11. Antibody responses to the M2e-HA1-HA2 peptide and individual epitopes

Sera collected from the experimental pigs were assessed for antibody responses against the M2e-HA1-HA2 peptide using an indirect ELISA. Briefly, 96-microwell ELISA plates (Corning, USA) were coated with 100 μl/well of 1:200 recombinant M2e-HA1-HA2 peptide (0.3 mg/ml in water) diluted in carbonate coating buffer (pH 9.6), and incubated overnight at room tempera-

Table 1
Lesion scores at necropsy on day 6 post-challenge.

Group	Gross Lesion Score/No of positive animals	Microscopic Lesions Score/No of positive animals	Immunohisto-Chemistry Score/No of positive animals	Total Lesion Score
PBS	24.000 ± 4.658 (5/5)	111.000 ± 11.692 (5/5)	56.000 ± 5.070 (5/5)	191.000 ± 19.967
$PEG_{600}PTHF_{650}$	15.000 ± 1.732 (5/5)	90.000 ± 2.550 (5/5)	26.000 ± 1.095[a] (5/5)	131.000 ± 1.924
M2e-HA1-HA2 Peptide	19.000 ± 3.701 (5/5)	97.000 ± 13.390 (5/5)	24.000 ± 3.421[a] (5/5)	140.000 ± 20.162
Vaccine ($PEG_{600}PTHF_{650}$/ M2e-HA1-HA2)	18.000 ± 2.966 (4/5)	69.000 ± 6.229 (4/5)	17.000 ± 3.130[a] (4/5)	97.000 ± 11.760[a]

Gross lesion scores – Total percentage of lungs affected (N = 5 pigs/group).
Microscopic lesion scores – Sum of the percentage of each lung section affected (N = 5 pigs/group, 6 lung sections per pig), scored as follows:
Bronchial/bronchiolar epithelial changes, and/or bronchitis and bronchiolitis - Scoring −25% airways affected = 1, 26–50% airways affected = 2 =, 51–75% airways affected = 3, 76–100% airways affected = 4.
Interstitial pneumonia (IP) - Scoring − 0 = none, 1 = mild, focal to multifocal IP, 2 = moderate, locally extensive to multifocal IP, 3 = moderate, multifocal to coalescing IP, 4 = severe, coalescing to diffuse.
Peribronchiolar lymphocytic cuffing − 0 = none, 1 = minimal, loosely formed, 2 = mild, loosely formed, 3 = moderate, well formed, 4 = severe, thick, well-formed cuffs.
Immunohistochemistry (IHC) scores – Sum of the number of sections positive for antigen as detected by a IAV-S specific monoclonal antibody and IHC score (N = 5 pigs/group, 6 lung sections per pig), Scoring - weak = 1, moderate = 2, strong = 3 (Fig. 6).
Total lesion scores – Sum of the gross, microscopic and IHC scores.
a- significantly different from the PBS group, b- significantly different from the $PEG_{600}PTHF_{650}$ group, c- significantly different from the M2e-HA1-HA2 peptide group. Mann-Whitney *U* test at $p \leq 0.05$.

ture, followed by five washes with PBST. Plates were blocked with 200 μl/well of blocking buffer (2% BSA in 1× PBST) for 2 h at 37 °C. After blocking, plates were washed five times using 1× PBST. To each well, 50 μl of 1:50 serum diluted in PBST was added in duplicate and incubated for 2 h at 37 °C. After washing five times with PBST, 50 μl/well of a 1:2500 diluted anti-swine IgG peroxidase-conjugated secondary antibody (KPL, USA) was added, and the plates were incubated at 37 °C for 1 h. After washing five times, 50 μl/well of TMB substrate (KPL, USA) was added to plates and incubated in the dark for 15 min at room temperature to catalyze the reaction. Finally, 50 μl/well of 1 M HCl was added to stop the reaction. The OD readings were obtained at 450 nm using a microplate reader (BioTek Instruments, Winooski, VT) (Fig. 4).

Antibody responses to the individual epitopes were measured essentially as described above, except that the test sera were blocked with commercial peptides (GenScript, Piscataway, NJ) encoding the epitopes before assessment on the ELISA. Each peptide (10ug) was added separately to the 1:50 dilution of the test sera and incubated at room temperature with gentle shaking for 1 h. The samples blocked by the synthetic peptides were then added to ELISA plate and OD values detected as described above. The antibody response to each peptide was measured as the reduction in OD value in comparison to the unblocked sample. Mean values of duplicate runs, normalized against a negative sample are presented in Fig. 5.

2.12. *Hemagglutination inhibition [41] assay*

The hemagglutination inhibition assay was performed using 0.5% chicken RBCs (Lampire Biological Laboratories, USA) and four hemagglutinating units of Influenza A/CA/04/09 H1N1 as per World Health Organization's protocol [30]. The collected serum samples were treated with receptor destroying enzyme (RDE) (Denka Seiken, USA) to remove nonspecific hemagglutination inhibitors. Forty-five microliters of RDE was added to the 15 μl of serum and incubated at 37 °C overnight, followed by RDE inactiva-

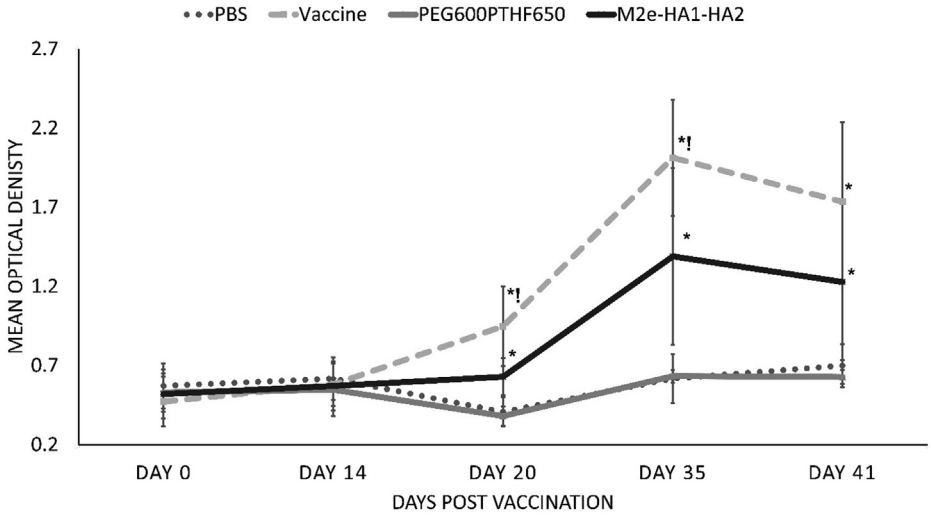

Fig. 4. Antibody response in vaccinated pigs. Antibody response against M2e-HA1-HA2 peptide measured as ELISA OD values. Y-axis - mean OD reading (450 nm) for each group, x-axis - days post vaccination (DPV). An asterisk (⋆) symbol represents the groups were statistically different ($p < 0.05$) from the PBS group at the respective days post vaccination (DPV). An exclamation (!) symbol represents $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 micellar assemblies vaccinated group is significantly different ($P < 0.05$, Student's T test) from M2e-HA1-HA2 peptide group at the respective time point.

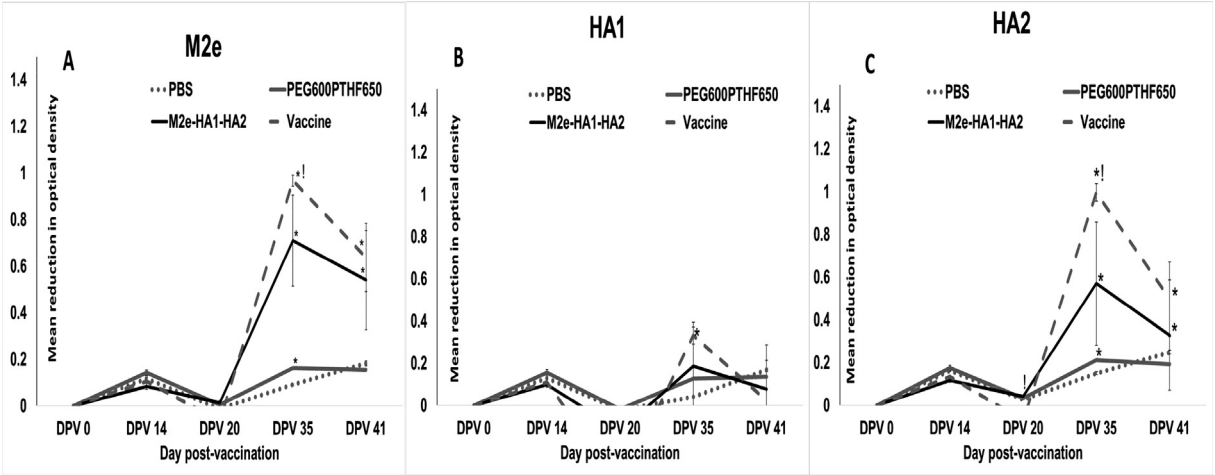

Fig. 5. Antibody responses to individual epitopes in vaccinated pigs. Antibody response against individual epitopes measured as a reduction in ELISA OD values using a blocking ELISA format. Y-axis - mean reduction in OD reading (450 nm) for each group compared to the unblocked sample, x-axis - days post vaccination (DPV). An asterisk (⋆) symbol represents the groups were statistically different ($p < 0.05$) from the PBS group at the respective days post vaccination (DPV). An exclamation (!) symbol represents $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 micellar assemblies vaccinated group is significantly different ($P < 0.05$, Student's T test) from M2e-HA1-HA2 peptide group at the respective time point.

tion at 56 °C for 30 min. The treated samples were diluted to 1:10 by adding 90 μl of PBS. Doubling dilutions were performed on the RDE treated serum, mixed thoroughly with 4 HA units of Influenza A (H1N1) pdm09 and incubated at 37 °C for 1 h. Following the incubation, 50 μl of 0.5% chicken RBCs (Lampire Biological Laboratories, USA) was added to each well, mixed by gentle vortexing and incubated for 15 min. The wells with button formations of RBCs were recorded as positive.

2.13. Detection of challenge virus shedding by qPCR

Virus shedding in nasal secretions was assessed by a diagnostic one-step qRT-PCR using a commercial kit; the Path-ID RT-PCR Kit (Thermo Fisher, USA) targeting the matrix gene. Viral nucleic acids were extracted from nasal swab samples with the MagMAX Total Nucleic Acid Isolation Kit (Thermo Fisher) and an automated extractor (Qiagen Biosprint 96) following the manufacturer's instructions. The standard curve was generated using log dilutions of a previously quantified virus culture and used to calculate the copy numbers. The assay was performed in duplicate by NDSU VDL, as per the manufacturer's instructions, following standardized operating procedures.

2.14. Statistical analysis

The antibody titers, HAI titer and the viral load in nasal secretions were compared by Student's *T*-test using Microsoft Excel 2016. The histology scores were compared by Mann-Whitney *U* test using SPSS software (IBM, USA). Data analysis were considered significant at $p < 0.05$.

3. Results

3.1. The peptide antigen interacts with the exterior of the micellar assemblies

The structure of PEG_{600}-$PTHF_{650}$ macromolecules contains 6 proton sites a, b, c, d, e and f, with d and f localizing to the hydrophobic PTHF fragments (Fig. 1, Panel 1). Proton spectra collected from solutions of 0.5 w/v% PEG_{600}-$PTHF_{650}$ alone or 5 w/v % polymer and 0.005% peptide assemblies in deuterated water showed that addition of peptide to the PEG_{600}-$PTHF_{650}$ micellar solution led to a shift of the signals for the hydrophilic PEG protons a and c (Fig. 1A and C). The broadening of the signals (half-height width increases from 2.8 Hz to 6.5 Hz for protons c) implied that the mobility of the PEG fragments became limited at those locations, presumably due to their interaction with M2e-HA1-HA2 molecules. The exterior of the polymer-peptide assemblies appeared to be more tightly packed as evidenced by signal broadening, due to the association of the polar fragments of the peptide at these zones, which is in agreement with previous studies for $PEG_{600}PTHF_{650}$ and two model peptides [27]. A slight shift of the signals of protons a and c toward lower ppm values indicated that the polarity in the micellar exterior became lower after polymer interaction with M2e-HA1-HA2, when compared with those of the micelles with no incorporated peptide molecules. The finding can be explained by the replacement of polar water molecules with the less polar hydrophilic fragments of M2e-HA1-HA2 upon peptide incorporation. After adding the M2e-HA1-HA2 peptide, the signals for protons b and e shifted upfield, implying changes in the microenvironmental polarity of the area where the protons b and e were localized. ^1H shift of the protons b and e corresponding to methylene group in the α position of the carbonyl groups in the PTHF moieties and succinic acid moieties respectively, indicated that these protons are transferred into a less polar micellar core (Fig. 1B and E). Addition of the peptide to the 0.5 w/v% solution of $PEG_{600}PTHF_{650}$ did not lead to chemical shifts of the signals of protons d and f attributed to the hydrophobic PTHF fragments (Fig. 1D and F). Hence, it can be concluded that the M2e-HA1-HA2 molecules are preferentially localized to the exterior of the $PEG_{600}PTHF_{650}$ micellar assemblies.

3.2. Micellar assemblies formed by 1 w/v% $PEG_{600}PTHF_{650}$ are efficient in peptide delivery

The purified M2e-HA1-HA2 peptide was detected at the expected molecular weight of 11Kd by a M2e-specific monoclonal antibody provided by Dr. Eileen Thacker, Iowa State University. No significant cytotoxicity was detected at any of the tested concentrations of 0.05, 0.2, 0.5, 0.75 and 1.0% w/v $PEG_{600}PTHF_{650}$ by the MTT assay compared to untreated cells (data not shown). Further assessment of the antigen loading capacity at the same concentrations of polymer showed that the 1 w/v% concentration of PEG_{600}-$PTHF_{650}$ had a significantly higher antigen loading capacity than the next lower dilution of 0.75 w/v% $PEG_{600}PTHF_{650}$ and the other dilutions tested (Fig. 2). As there was also no significant cytotoxicity at the 1 w/v% $PEG_{600}PTHF_{650}$, this concentration was used for further testing and vaccine formulation (Fig. 2).

When the effectiveness of intracellular delivery of the incorporated peptide was assessed by an immunofluorescence assay (IFA), the control recombinant M2e-HA1-HA2 peptide could not penetrate cells by itself as it is highly hydrophobic in nature and, thus, poorly water-soluble. As expected, no signal was detected with the polymer alone. It could only be internalized with the help of delivery vehicle. Increasing the incubation time from 1 to 8 h led to proportional increases in delivery as observed in the signal intensity in the IFA for the of the 1% w/v micellar assemblies (Fig. 3).

3.3. Vaccination induces strong antibody responses against the peptide antigen

Piglets vaccinated with $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 micelles and M2e-HA1-HA2 peptide alone mounted strong antibody responses against the M2e-HA1-HA2 peptide. The antibody titers levels increased with the boosters on DPV 20 and 35. The mean optical density values remained significantly different ($P < 0.05$) from pigs vaccinated with PBS and $PEG_{600}PTHF_{650}$ micelles alone for the duration of the study. Piglets vaccinated with PEG_{600}-$PTHF_{650}$/M2e-HA1-HA2 micellar assemblies mounted significantly stronger antibody responses when compared to M2e-HA1-HA2 peptide alone on DPV 20 and DPV 35 indicating that $PEG_{600}PTHF_{650}$ acts as an adjuvant by enhancing antibody responses (Fig. 4). Antibody responses to all three epitopes were detected in vaccinated pigs, pre-challenge. However, the responses to the HA1 peptide were low (Fig. 5B). At DPV 35, pigs administered $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 had higher levels of each peptide specific antibody than pigs immunized with the M2e-HA1-HA2 peptide alone. As expected the unvaccinated and $PEG_{600}PTHF_{650}$ groups did not show significant responses to the individual peptides (Fig. 5).

The haemagglutination inhibition (HAI) titers for all piglets for the duration of the study were < 40, with no significant differences between groups [data not shown], suggesting that the antibodies generated against the M2e-HA1-HA2 peptide were not neutralizing in nature.

3.4. Vaccination reduces lung pathology

No clinical signs of IAV infection such as pyrexia, respiratory distress or body weight loss was observed in any of the piglets throughout the study. When necropsy was conducted on the 6th day post challenge, 1 of the 5 $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 vacci-

nated pigs did not develop any gross or microscopic lesions (Table 1). The unvaccinated pigs had a total microscopic lesion score of 111.00 while the vaccinated pigs had a score of 69.00 (Table 1). While the gross and microscopic lesion scores for the vaccinated and control pigs immunized with the peptide alone were lesser than those of the unvaccinated pigs, these differences were not statistically significant. However, the amount of viral antigen detected by IHC was significantly different between the unvaccinated and vaccinated pigs. The total consolidated lesion score for the unvaccinated group (191.00) was significantly different from that of the vaccinated pigs (97.00). While the total scores for the peptide (141.00) and polymer (130.00) groups were considerably lower than those of the unvaccinated pigs but higher than the $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 vaccinated pigs (97.00), statistical significance was not detected by the Mann Whitney *U* test (Table 1).

3.5. Vaccination induces delayed but significant reduction of viral shedding

Protection against nasal shedding of the challenge virus by vaccination as measured with a matrix gene-specific qPCR unexpectedly showed that viral loads in pigs vaccinated with the peptide alone or the polymer-peptide vaccine were significantly higher than the PBS control group, at 3 days post challenge (DPC 3). However, values for the pigs administered the polymer alone were similar to those of the PBS control group, indicating that early enhancement of viral replication was due to the peptide vaccine construct and not the AIP-based delivery system. Three days later, on the 6th day post challenge, the trend reversed to where nasal shedding of the challenge virus was significantly lower in the pigs administered the $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 vaccine and peptide alone compared to unvaccinated pigs, while the viral loads continued to increase in pigs administered PBS or the polymer alone. The difference in viral loads between DPC 3 and DPC 6 in the pigs administered either the peptide alone or the polymer-peptide vaccine were statistically different, indicating that influenza-specific protection induced by vaccination was delayed but robust, resulting in a significant reduction in challenge viral shedding during the 3 days period (Fig. 6).

3.6. The $PEG_{600}PTHF_{650}$ peptide vaccine was safe

No untoward clinical signs were observed in any of the animals prior to challenge. Similarly, no gross or microscopic lesions were observed in the vaccinated piglets euthanized prior to challenge, indicating the $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 vaccine did not cause any side effects. Localization of the M2e-HA1-HA2 peptide by immunohistochemistry using the M2e peptide-specific monoclonal antibody showed antigen-specific staining in the alveolar septa, alveolar spaces and perivascular areas of the lung tissue and lymph node sections. Representative images are depicted in Fig. 7. This

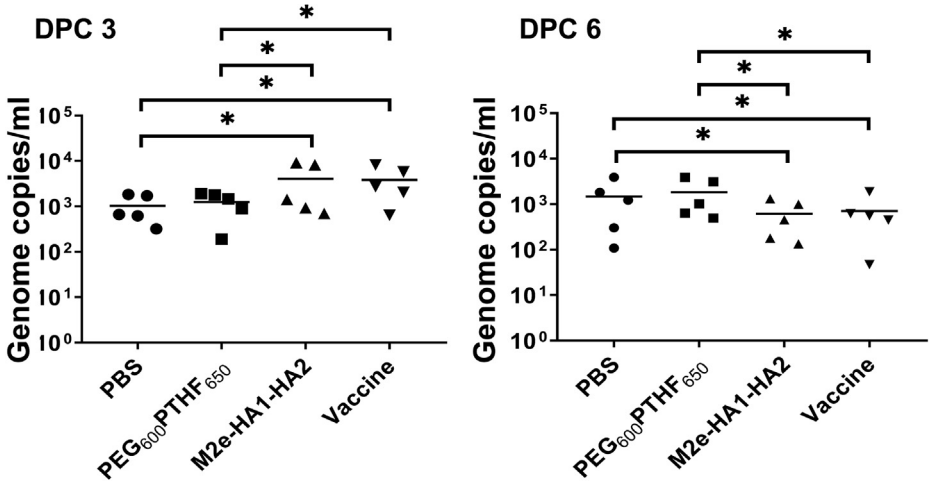

Fig. 6. Detection of viral load in nasal secretions. The challenge pH1N1 viral particles in nasal secretions swabs for each treatment group on day 3 and 6 post-challenge (DPC) were determined by qRT-PCR. X-Axis – Groups, Y-axis – Mean genomic copies/ml in individual pig. An asterisk (*) symbol represents the groups were statistically different ($p < 0.05$) from each other. Horizontal bars represents the mean genomic copies/ml in the group.

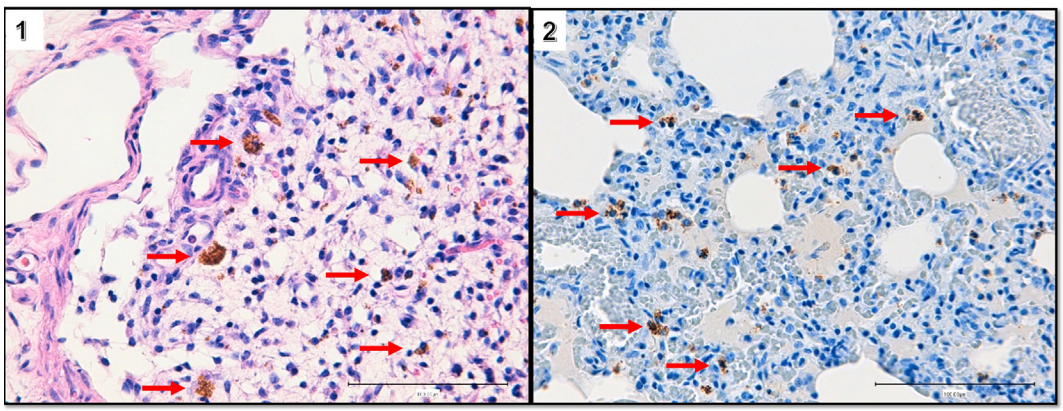

Fig. 7. Localization of vaccine antigen in pigs vaccinated with $PEG_{600}PTHF_{650}$/M2e-HA1-HA2. 1-Lymph node, 2-Lung. Arrows indicate brown staining of the peptide antigen detected by a M2e-specific monoclonal antibody. No antigen was detected in unvaccinated pigs (data not shown).

observation suggests that $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 complexes were taken up by antigen presenting cells and transported to the germinal center in lymph nodes. Antigen specific staining was absent in the PBS control pigs sacrificed prior to challenge.

4. Discussion

The discovery that the highly conserved M2e and HA2 epitopes can confer broad protection against influenza viruses was a major breakthrough in the development of universal vaccines against influenza viruses [8]. These epitopes have been tested extensively and successfully in mice; individually or in conjunction with other immunogenic proteins and peptides [3,5,7,8,13,42,43]. However, in pig models the M2e peptide failed to reduce challenge virus shedding and ameliorate disease outcomes [3,5–8,13–16,42,43]. A combination of the M2e and HA2 epitopes, together with a H1N1strain-specific HA1 epitope, as used in this study, has not been tested before in pigs to determine if there are synergistic protective effects. Similarly, this study addresses the need for effective delivery systems for peptide antigens which are inherently poor immunogens [16,44] but have great promise in inducing epitope-specific, broad coverage. Our results support our hypothesis that the micellar assemblies prepared from amphiphilic invertible polymers (AIPs), represented in this study by $PEG_{600}PTHF_{650}$, effectively deliver the peptide vaccine cargo and act as an adjuvant in stimulating a strong humoral immune response against the delivered peptide.

While several conserved influenza epitopes have been identified, the M2e and HA2 epitopes are the most widely tested [4,5,8–10,35]. The HA2 epitope is an 11 amino acid conserved sequence in the N-terminal of the HA2 subunit of HA protein. In mouse models, vaccination with the HA2 peptide provided complete protection against IAV; whereas, this has not been studied yet in pig models [45,46]. However, when swine were vaccinated with an inactivated human H1N2 whole virus preparation and challenged with Influenza A (H1N1) pdm09, strong antibody responses to the HA2 fusion protein region but not the receptor binding region were elicited, and enhanced infection of MDCK cells with the pH1N1 virus by promoting viral membrane fusion [47]. The M2e epitope is a 23 amino acid long, highly conserved peptide chain from the M2 protein. M2e-based vaccine was also completely protective against multiple IAV strains in mice models [4,5,9,10], whereas, in ferrets [48] and chicken [49,50] M2e vaccination was only able to reduce the virus shedding and pathological symptoms. However, in pigs M2e-based vaccines resulted in enhanced challenge viral infection [14,15]. The strain-specific HA1 epitope used in this study was untested in swine but reduced viral shedding and pathology in a mouse model [35]. While the experimental conditions in our study do not exactly match those referenced above, the early enhancement of viral replication seen in the peptide-immunized animals was similar to observations in other studies cited above. Previous studies in mice suggest that the enhancement of infection in mice immunized with a chimeric peptide encoding the M2e and HA2 epitopes occurs via Fc region-based antibody dependent enhancement of infection of macrophages [11,51].

Although viral RNA loads were significantly different between vaccinated and unvaccinated animals at DPC 6, since virus load in the nasal secretions was measured by qRT-PCR and not a replicative assay, the possibility that replicating virus titers might be similar in vaccinated versus non-vaccinated groups cannot be ruled out. While the detailed characterization of the mechanisms involved is not within the scope of this study, our hypothesis that a combination of the AIP packaged epitopes will improve protection against influenza infection is well supported by the similar trends in the pathology and viral load data showing lower values for vaccinated animals. While the protection seen in this study can also be attributed to differences in the vaccine construct, dose, route of vaccination and infection, challenge virus strain and culture conditions used [52–56], further improvements in design, possibly by the addition of other B or T cell epitopes, could further improve the early immune responses and viral clearance.

Several approaches such as linking epitopes with immunogenic peptides or proteins, using multiple copies of epitopes, creating virus-like particles or using amphiphilic polymers [3–6,8,18,25] have been previously used to improve the weak immunogenicity of peptide antigens [16,44]. Amphiphilic polymers have several advantages; they can form micelles and micellar assemblies that can load antigen in a controllable manner, can be used for controlled antigen release and are generally immunologically safe [57]. Efficient entrapment and delivery of the hydrophobic drug, curcumin, into cancerous breast carcinoma and osteosarcoma cells as a potential treatment for breast and bone cancer respectively, was previously demonstrated for the AIP macromolecules used as the vaccine delivery vehicle in this study [58]. Interestingly, unlike previous studies [28,29], the peptide molecules are preferentially localized within the PEG exterior of the $PEG_{600}PTHF_{650}$ micellar assemblies, which can be attributed to the higher molecular weight of the M2e-HA1-HA2 peptide (11 Kd, compared to the 1.6–1.9 Kd for peptides studied in [28,29]), differences in peptide sequence and conformation of the molecules. While there is no previously published data on the possible biological mechanisms of action of the AIP, it is evident that incorporation of M2e-HA1-HA2 peptide in aqueous solution into micellar assemblies resulted in protection and effective delivery of the antigen vaccination. While the peptide alone was not uptaken by Vero cells due to its hydrophobic nature the micellar assemblies clearly enhanced the bioavailability and delivery of the M2e-HA1-HA2 peptide into cells in vitro (Fig. 3) and in vivo (Fig. 7) Since the M2e-HA1-HA2 peptide was found in the lymph nodes, the AIP micellar assemblies most likely faciltiated peptide uptake by antigen presenting cells. The adjuvant effects in enhancing antibody mediated immunity are clearly substantiated by the significantly higher peptide-specific antibody titers in piglets vaccinated with $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 micellar assemblies compared to control piglets vaccinated with M2e-HA1-HA2 peptide alone. Further, as no toxicity was noted in vitro or in vivo, and strong antibody responses were elicited aginst the peptide, the $PEG_{600}PTHF_{650}$ based delivery system could be potentially promising as a peptide antigen delivery system. The AIP's efficacy in delivering hydrophilic peptides or other complex peptides with diverse physical properties remains to be tested.

M2e antibodies can prevent the release of viral RNA genome from the endosome by preventing ion channel activity of the M2 protein. HA2 antibodies are reported to bind with the fusion peptide of HA2 protein hence preventing the fusion of the viral envelope with the endosomal membrane [59]. Hence, anti-M2e and HA2 antibodies are non-neutralizing and likely do not prevent virus attachment and initial infection [11,13,15,16] but likely act via other mechanisms which are not fully understood. Studies in mice suggest that anti-M2e and anti-HA2 antibodies could reduce the viral replication by eliminating infected cells by antibody dependent cell-mediated phagocytosis [43]. While characterization of cell mediated or innate immune mechanisms was not undertaken in this study, similar mechanisms were likely involved in this study, as animals vaccinated with $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 micellar assemblies had fewer lung lesions compared to the control groups in the absence of neutralizing antibody responses. The HA1 epitope-specific antibodies could be expected to bind to the cleavage site of precursor HA0 protein thus prevent the formation of HA1-HA2 mature protein [35] and can be expected to have a

neutralizing effect. Perhaps because antibody responses to this peptide were low in vaccinated pigs, neutralizing antibody responses were not detected in-vitro, in this study. Other limitations of this study are that the level of IgA antibodies were not determined and protection was assessed only against the Influenza A (H1N1) pdm09 strain.

In conclusion, the use of multiple epitopes and the adjuvant effects of the micellar assemblies from AIP macromolecules were effective in enhancing epitope-based immunization approaches against influenza viruses. A more detailed characterization of the mechanisms by which the $PEG_{600}PTHF_{650}$ assemblies modulate immunity will help to fully exploit its use as a delivery system and adjuvant.

Acknowledgements

We thank Dr. Eileen Thacker, director of the U.S National Poultry Research Center, Athens, GA for the M2e-specific monoclonal antibody. We would like to thank the staff of the North Dakota State University Veterinary Diagnostic Laboratory and South Dakota State University Veterinary Diagnostic Laboratory for their technical assistance. We are especially grateful to Dr. Michele Mucciante, Amanda Zubke and Gregg Baumann for assistance with the animal experimentation. We thank Ms. Heather Vinson, Scott Hosselton and Marvin Ssemadaali for proof-reading the manuscript.

Declaration of Competing Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclose receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by the Agricultural Products Utilization Committee (APUC) of N. Dakota, the N. Dakota State Board of Agricultural Research (SBARE).

References

[1] Imai M, Kawaoka Y. The role of receptor binding specificity in interspecies transmission of influenza viruses. Curr Opin Virol 2012;2:160–7.
[2] Ma W, Lager K, Vincent A, Janke B, Gramer M, Richt J. The role of swine in the generation of novel influenza viruses. Zoonoses Public Health 2009;56:326–37.
[3] De Filette M, Min Jou W, Birkett A, Lyons K, Schultz B, Tonkyro A, et al. Universal influenza A vaccine: optimization of M2-based constructs. Virology 2005;337:149–61.
[4] De Filette M, Fiers W, Martens W, Birkett A, Ramne A, Lowenadler B, et al. Improved design and intranasal delivery of an M2e-based human influenza A vaccine. Vaccine 2006;24:6597–601.
[5] De Filette M, Ramne A, Birkett A, Lycke N, Lowenadler B, Min Jou W, et al. The universal influenza vaccine M2e-HBc administered intranasally in combination with the adjuvant CTA1-DD provides complete protection. Vaccine 2006;24:544–51.
[6] Huleatt JW, Nakaar V, Desai P, Huang Y, Hewitt D, Jacobs A, et al. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine 2008;26:201–14.
[7] Fiers W, De Filette M, El Bakkouri K, Schepens B, Roose K, Schotsaert M, et al. M2e-based universal influenza A vaccine. Vaccine 2009;27:6280–3.
[8] Du L, Zhou Y, Jiang S. Research and development of universal influenza vaccines. Microbes Infect 2010;12:280–6.
[9] Zhao G, Lin Y, Du L, Guan J, Sun S, Sui H, et al. An M2e-based multiple antigenic peptide vaccine protects mice from lethal challenge with divergent H5N1 influenza viruses. Virol J 2010;7:9.
[10] Wen F, Ma J-H, Yu H, Yang F-R, Huang M, Zhou Y-J, et al. A novel M2e-multiple antigenic peptide providing heterologous protection in mice. J Vet Sci 2016;17:71–8.
[11] Ameghi A, Pilehvar-Soltanahmadi Y, Baradaran B, Barzegar A, Taghizadeh M, Zarghami N, et al. Protective immunity against homologous and heterologous influenza virus lethal challenge by immunization with new recombinant chimeric HA2-M2e fusion protein in BALB/C mice. Viral Immunol 2016;29:228–34.
[12] Wu K-W, Chien C-Y, Li S-W, King C-C, Chang C-H. Highly conserved influenza A virus epitope sequences as candidates of H3N2 flu vaccine targets. Genomics 2012;100:102–9.
[13] Staneková Z, Varečková E. Conserved epitopes of influenza A virus inducing protective immunity and their prospects for universal vaccine development. Virology J 2010;7:351.
[14] Opriessnig T, Gauger PC, Gerber PF, Castro AM, Shen H, Murphy L, et al. Comparison of the efficacy of a commercial inactivated influenza A/H1N1/pdm09 virus (pH1N1) vaccine and two experimental M2e-based vaccines against pH1N1 challenge in the growing pig model. PLoS ONE 2018;13:e0191739.
[15] Heinen PP, Rijsewijk FA, de Boer-Luijtze EA, Bianchi AT. Vaccination of pigs with a DNA construct expressing an influenza virus M2–nucleoprotein fusion protein exacerbates disease after challenge with influenza A virus. J Gen Virol 2002;83:1851–9.
[16] Chun S, Li C, Van Domselaar G, Wang J, Farnsworth A, Cui X, et al. Universal antibodies and their applications to the quantitative determination of virtually all subtypes of the influenza A viral hemagglutinins. Vaccine 2008;26:6068–76.
[17] Daniels R, Downie J, Hay A, Knossow M, Skehel J, Wang M, et al. Fusion mutants of the influenza virus hemagglutinin glycoprotein. Cell 1985;40:431–9.
[18] Stanekova Z, Kiraly J, Stropkovska A, Mikušková T, Mucha V, Kostolanský F, et al. Heterosubtypic protective immunity against influenza A virus induced by fusion peptide of the hemagglutinin in comparison to ectodomain of M2 protein. Acta Virol 2011;55:61–7.
[19] Wang TT, Tan GS, Hai R, Pica N, Ngai L, Ekiert DC, et al. Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. Proc Natl Acad Sci 2010;107:18979–84.
[20] Jang Y, Seong B. Options and obstacles for designing a universal influenza vaccine. Viruses 2014;6:3159–80.
[21] Fosgerau K, Hoffmann T. Peptide therapeutics: current status and future directions. Drug Discov Today 2015;20:122–8.
[22] Moghimi SM, Hunter AC. Poloxamers and poloxamines in nanoparticle engineering and experimental medicine. Trends Biotechnol 2000;18:412–20.
[23] Newman MJ, Todd CW, Balusubramanian M. Design and development of adjuvant-active nonionic block copolymers. J Pharm Sci 1998;87:1357–62.
[24] Greenland JR, Letvin NL. Chemical adjuvants for plasmid DNA vaccines. Vaccine 2007;25:3731–41.
[25] Adams JR, Haughney SL, Mallapragada SK. Effective polymer adjuvants for sustained delivery of protein subunit vaccines. Acta Biomater 2015;14:104–14.
[26] Voronov A, Kohut A, Peukert W, Voronov S, Gevus O, Tokarev V. Invertible architectures from amphiphilic polyesters. Langmuir 2006;22:1946–8.
[27] Voronov A, Kohut A, Vasylyev S, Peukert W. Mechanism of silver ion reduction in concentrated solutions of amphiphilic invertible polyesters in nonpolar solvent at room temperature. Langmuir 2008;24:12587–94.
[28] Kohut A, Zholobko O, Hevus I, Voronov A. 1H NMR Study of "host–guest" interactions of micellar assemblies from amphiphilic invertible polymers and peptides. Macromol Chem Phys 2017;218.
[29] Pan Y, Zholobko O, Voronov A, Yang Z. Inversion of polymeric micelles probed by spin labeled peptide incorporation and electron paramagnetic resonance. J Phys Chem C 2018.
[30] Organization WH. Manual for the laboratory diagnosis and virological surveillance of influenza; 2011.
[31] Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints 1 2. Am J Epidemiol 1938;27:493–7.
[32] Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat Med 1999;5:1157.
[33] Hashem AM, Van Domselaar G, Li C, Wang J, She Y-M, Cyr TD, et al. Universal antibodies against the highly conserved influenza fusion peptide cross-neutralize several subtypes of influenza A virus. Biochem Biophys Res Commun 2010;403:247–51.
[34] Nagy Z, Rajnavölgyi E, Hollosi M, Tóth G, Váradi G, Penke B, et al. The intersubunit region of the influenza virus haemagglutinin is recognized by antibodies during infection. Scand J Immunol 1994;40:281–91.
[35] Horváth A, Tóth GK, Gogolák P, Nagy Z, Kurucz I, Pecht I, et al. A hemagglutinin-based multipeptide construct elicits enhanced protective immune response in mice against influenza A virus infection. Immunol Lett 1998;60:127–36.
[36] Hevus I, Kohut A, Voronov A. Interfacial micellar phase transfer using amphiphilic invertible polymers. Polym Chem 2011;2:2767–70.
[37] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[38] Opriessnig T, Patterson AR, Madson DM, Pal N, Ramamoorthy S, Meng XJ, et al. Comparison of the effectiveness of passive (dam) versus active (piglet) immunization against porcine circovirus type 2 (PCV2) and impact of passively derived PCV2 vaccine-induced immunity on vaccination. Vet Microbiol 2010;142:177–83.

[39] Halbur P, Paul P, Frey M, Landgraf J, Eernisse K, Meng X-J, et al. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet Pathol 1995;32:648–60.
[40] Gauger PC, Vincent AL, Loving CL, Henningson JN, Lager KM, Janke BH, et al. Kinetics of lung lesion development and pro-inflammatory cytokine response in pigs with vaccine-associated enhanced respiratory disease induced by challenge with pandemic (2009) A/H1N1 influenza virus. Vet Pathol 2012;49:900–12.
[41] Ellis J, Hassard L, Clark E, Harding J, Allan G, Willson P, et al. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Canad Veterin J = La revue veterinaire canadienne 1998;39:44–51.
[42] Adar Y, Singer Y, Levi R, Tzehoval E, Perk S, Banet-Noach C, et al. A universal epitope-based influenza vaccine and its efficacy against H5N1. Vaccine 2009;27:2099–107.
[43] El Bakkouri K, Descamps F, De Filette M, Smet A, Festjens E, Birkett A, et al. Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection. J Immunol 2011;186:1022–31.
[44] Feng J, Zhang M, Mozdzanowska K, Zharikova D, Hoff H, Wunner W, et al. Influenza A virus infection engenders a poor antibody response against the ectodomain of matrix protein 2. Virol J 2006;3:102.
[45] Bommakanti G, Citron MP, Hepler RW, Callahan C, Heidecker GJ, Najar TA, et al. Design of an HA2-based Escherichia coli expressed influenza immunogen that protects mice from pathogenic challenge. Proc Natl Acad Sci 2010;107:13701–6.
[46] Steel J, Lowen AC, Wang TT, Yondola M, Gao Q, Haye K, et al. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. MBio 2010;1: e00018–e110.
[47] Khurana S, Loving CL, Manischewitz J, King LR, Gauger PC, Henningson J, et al. Vaccine-induced anti-HA2 antibodies promote virus fusion and enhance influenza virus respiratory disease. Sci Transl Med 2013;5. 200ra114-200ra114.
[48] Fan J, Liang X, Horton MS, Perry HC, Citron MP, Heidecker GJ, et al. Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. Vaccine 2004;22:2993–3003.
[49] Zhang X, Liu M, Liu C, Du J, Shi W, Sun E, et al. Vaccination with different M2e epitope densities confers partial protection against H5N1 influenza A virus challenge in chickens. Intervirology 2011;54:290–9.
[50] Reese KA, Lupfer C, Johnson RC, Mitev GM, Mullen VM, Geller BL, Pastey M. A novel lactococcal vaccine expressing a peptide from the M2 Antigen of H5N2 Highly Pathogenic Avian Influenza a virus prolongs survival of vaccinated chickens. Veterin Med Int 2013;2013:1–8. https://doi.org/10.1155/2013/316926.
[51] Dutry I, Yen H, Lee H, Peiris M, Jaume M. Antibody-Dependent Enhancement (ADE) of infection and its possible role in the pathogenesis of influenza. BMC Proc 2011:P62.
[52] Mozdzanowska K, Zharikova D, Cudic M, Otvos L, Gerhard W. Roles of adjuvant and route of vaccination in antibody response and protection engendered by a synthetic matrix protein 2-based influenza A virus vaccine in the mouse. Virol J 2007;4:118.
[53] Price GE, Lo C-Y, Misplon JA, Epstein SL. Reduction of influenza virus transmission from mice immunized against conserved viral antigens is influenced by route of immunization and choice of vaccine antigen. Vaccine 2018;36:4910–8.
[54] Schepens B, De Vlieger D, Saelens X. Vaccine options for influenza: thinking small. Curr Opin Immunol 2018;53:22–9.
[55] Kim Y-J, Lee Y-T, Kim M-C, Lee Y-N, Kim K-H, Ko E-J, et al. Cross-protective efficacy of influenza virus M2e containing virus-like particles is superior to hemagglutinin vaccines and variable depending on the genetic backgrounds of mice. Front Immunol 2017;8:1730.
[56] Wolf AI, Mozdzanowska K, Williams KL, Singer D, Richter M, Hoffmann R, et al. Vaccination with M2e-based multiple antigenic peptides: characterization of the B cell response and protection efficacy in inbred and outbred mice. PLoS ONE 2011;6:e28445.
[57] Shakya AK, Nandakumar KS. Applications of polymeric adjuvants in studying autoimmune responses and vaccination against infectious diseases. J R Soc Interface 2013;10:20120536.
[58] Hevus I, Modgil A, Daniels J, Kohut A, Sun C, Stafslien S, et al. Invertible micellar polymer assemblies for delivery of poorly water-soluble drugs. Biomacromolecules 2012;13:2537–45.
[59] Imai M, Sugimoto K, Okazaki K, Kida H. Fusion of influenza virus with the endosomal membrane is inhibited by monoclonal antibodies to defined epitopes on the hemagglutinin. Virus Res 1998;53:129–39.