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Synthesis and Immunogenicity Assessment of Elastin-Like Polypeptide-M2e Construct as an Influenza Antigen

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Abstract

The 23 amino acid-long extracellular domain of the influenza virus transmembrane protein M2 (M2e) has remained highly conserved since the 1918 pandemic, and is thus considered a good candidate for development of a universal influenza A vaccine. However, M2e is poorly immunogenic. In this study we assessed the potential of increasing immunogenicity of M2e by constructing a nanoscale-designed protein polymer containing the M2e sequence and an elastin-like polypeptide (ELP) nanodomain consisting of alanine and tyrosine guest residues (ELP(A₂YA₂)₂₄). The ELP nanodomain was included to increase antigen size, and to exploit the inherent thermal inverse phase transition behavior of ELPs to purify the protein polymer. The ELP(A₂YA₂)₂₄ + M2e nanodomain molecule was recombinantly synthesized. Characterization of its inverse phase transition behavior demonstrated that attachment of M2e to ELP(A₂YA₂)₂₄ increased its transition temperature compared to ELP(A₂YA₂)₂₄. Using a dot blot test we determined that M2e conjugated to ELP is recognizable by M2e-specific antibodies, suggesting that the conjugation process does not adversely affect the immunogenic property of M2e. Further, upon vaccinating mice with ELP(A₂YA₂)₂₄ + M2e it was found that indeed the nanodomain protein enhanced M2e-specific antibodies in mouse serum compared to free M2e peptide and ELP(A₂YA₂)₂₄. The immune serum could also recognize M2 expressed on influenza virions. Overall, this data suggests the potential of using molecules containing M2e-ELP nano-domains to develop a universal influenza vaccine.

Keywords

Elastin-like polypeptide; influenza vaccine; M2e fusion protein; M2e vaccine; universal influenza vaccine

1. Introduction

Influenza virus is one of the most deadly human pathogen. The world has already witnessed four pandemics caused by human influenza. The first recorded pandemic was in 1918 (H1N1 Spanish influenza), which claimed lives of 50 million people worldwide. The second and third pandemics of 1957 (H2N2 Asian influenza) and 1968 (H3N2 Hong Kong influenza), respectively, claimed over a million lives each, around the world.¹ The fourth influenza pandemic, the first in the 21st century was reported in 2009. It originated with an outburst of H1N1 influenza virus of swine origin in the southern part of United States and Mexico resulting in 8000– 18000 deaths worldwide.² Even seasonal influenza epidemics lead to remarkable morbidity and mortality throughout the globe with about 3–5 million cases of severe influenza infection and an estimated 500 000 deaths worldwide each year.³

Vaccination provides high degree of protection from influenza infection.⁴ Current influenza vaccines rely on antibody induction directed against two viral surface glycoproteins — hemagglutinin (HA) and neuraminidase (NA). In order for the vaccine to be effective, the virus strain should match the strains used to develop the vaccine. Unfortunately, influenza virus exhibits high variability in its genomic make up. For example, selection pressure to evade human immunity coupled with the naturally error-prone RNA polymerase causes the virus to undergo point mutations within epitope sequences of antigenic glycoproteins — HA and NA, giving rise to a progeny with a slightly different genetic make-up (antigenic drift).⁵ Furthermore, genetic reassortment can also occur when two strains of influenza circulate together in a suitable host. If exchange of genetic material was to occur between a subtype that can infect humans and one that can infect another species, the resulting strain could have pandemic potential. This genomic plasticity of influenza virus, which results in ever-changing HA and NA molecules, makes it tough to control influenza virus just based on the current vaccine design.⁶

To overcome this drawback of existing vaccines, universal influenza vaccines are being developed that can potentially provide a broader protection against current and emergent influenza A strains. One such candidate for universal vaccine development is the extracellular domain of the viral transmembrane protein M2 (M2e), whose 23 amino acid sequence has remained highly conserved since the 1918 pandemic.⁷ However, M2e is poorly immunogenic, because it is present in small numbers on the viral surface, and its small size causes it to be shielded by the much larger surface glycoproteins, HA and NA, reducing its visibility to the immune cells.^{8,9} Indeed studies have shown that humans after natural influenza infections have very little to no serum antibodies against M2.^{10,11} It has been found that immunogenicity of M2e can be greatly enhanced by linking M2e to an appropriate carrier. Different carriers that have been investigated thus far to increase M2e immunogenicity include hepatitis B virus core subunit (HBc),¹² flagellin,¹³ norovirus P particle,⁵ *Neisseria meningitidis* outer membrane protein complex,¹⁰ bovine serum albumin,¹⁰ keyhole limpet hemocyanin,¹⁴ virus-like particles,^{15,16} phage Q- β ,¹⁷ human papillomavirus¹⁸ and papaya mosaic virus.¹⁹

In this study, we constructed a protein polymer containing the M2e sequence and an elastin-like polypeptide (ELP) nanodomain. ELPs contain a repeat sequence of GXGVP, where “X”

can be any amino acid except proline.^{20,21} ELPs are thermally sensitive and exhibit inverse phase transition behavior: they remain soluble in water below an inverse transition temperature (T_i) but precipitate when the temperature is raised above T_i .^{20,22} Composition of guest residue “X” in GXGVP can play an important role in deciding the T_i for a given ELP.²¹ ELPs are biocompatible and biodegradable, and have thus attracted much attention for drug delivery and tissue engineering applications.^{20,22–26} More recently, ELPylation, the process of recombinantly fusing proteins to ELP has been used to purify proteins including influenza antigens,^{27,28} *Mycobacterium tuberculosis* antigens,²⁹ antibody fragments³⁰ and complete antibodies.³¹ Protein purification is simply achieved by cycling the ELPylated protein solution above the T_i to cause protein precipitation, centrifugation to remove the contaminated supernatant, and then lowering the temperature below T_i to redissolve the ELPylated protein molecules in fresh buffer. Repeated cycles result in purification of the ELPylated proteins without much loss in yield.³² The process of ELPylation has no adverse effect on the activity of recombinant protein, and ELP does not interfere with the biological processes such as folding and post-translational modification of the recombinant protein.³¹ ELPylation tends to enhance the stability of the recombinant protein and it has been seen that the recombinant proteins produced with ELP are capable of generating immune response indicating no degradation of the epitope.²⁹

We postulated that by utilizing the ELPylation strategy, M2e peptide could be recombinantly fused with ELP nanodomain to create an ELP + M2e nanoscale-designed protein polymer. Because ELP + M2e is expected to be much larger as compared to M2e peptide alone, we reasoned that the new nanoscale-designed protein polymer may exhibit increased immunogenicity, similar to that when M2e is attached to a carrier protein such as albumin or keyhole limpet hemocyanin. Accordingly, in this study we demonstrate the synthesis, purification and characterization of M2e fused to ELP nanodomains with “alanine” and “tyrosine” as the guest residues. Tyrosine was selected to make the ELP molecule hydrophobic since it has previously been shown that synthetic block copolymers with higher hydrophobic content exhibit a higher adjuvant property.^{33,34} Finally, *in vivo* immunogenicity of ELP + M2e nanoscale-designed protein polymer was determined and compared against free M2e peptide.

2. Experimental Section

2.1. Materials

Custom oligonucleotides coding for pET-24 a (+)-modifier insert, ELP monomer sequence and M2e were synthesized by Integrated DNA Technologies Inc. (IA, USA). Restriction enzymes — BamHI, XbaI, AclI, BseRI and BglII; alkaline phosphatase; T4 polynucleotide kinase (3' phosphatase minus) and T4 DNA ligase were obtained from New England Biolabs (MA, USA). pET-24 a (+) cloning vector was purchased from Novagen Inc. (WI, USA). NEB 10-beta competent *Escherichia coli* (high efficiency) cells, and BL21 (DE3) competent *E. coli* cells were purchased from New England Biolabs (MA, USA). The cell cultures were grown in a magnificent broth medium which was purchased from MacConnell Research (CA, USA). Sodium chloride (NaCl) was purchased from Fisher Scientific (PA, USA). PCR purification kit and DNA miniprep kit were purchased from QIAGEN Inc.

(MD, USA). DNA gel purification kit was obtained from Promega (WI, USA). Goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) was purchased from Southern Biotech (AL, USA). Tween® 20 was obtained from Fisher Scientific (PA, USA). Phosphate-citrate buffer tablet was purchased from Sigma-Aldrich (MO, USA). *O*-Phenylenediamine (OPD) was purchased from Invitrogen (NY, USA).

3. Methods

3.1. Protein synthesis

Modification of DNA and synthesis of genes was done by following the standard protocols of molecular biology. As a first step in constructing a recombinant vector for ELP expression under T-7 promoter, a modifier insert sequence was introduced into expression vector pET-24 a(+).²¹ Next, synthetic genes for ELP monomer were assembled and plasmid reconstruction recursive directional ligation (PRe-RDL) described elsewhere²¹ was used to sequentially introduce ELP-coding chain with desired number of ELP monomer repeats in to the modified pET-24 a(+) vector. The monomer of ELP has a sequence of GXGVP with alanine and tyrosine in a ratio of 4:1 i.e., [(GAGVP)₂-(GYGVP)-(GAGVP)₂]. Henceforth, for convenience, the nomenclature used to describe ELP is ELP(A₂YA₂)_z, where *z* = number of repeats of monomer gene of an ELP. Once the desired number of ELP repeats were introduced in to the modified pET-24 a(+) vector, M2e gene sequence was inserted at the N-terminus of the ELP. At each step, number of monomer gene repeats and the DNA sequence was confirmed by DNA sequencing (3130 Genetic analyzer, Applied Biosystems, Center for Biotechnology and Genomics, Texas Tech University, TX, USA).

3.2. Protein expression

Purified and sequence-confirmed plasmids were then transformed into BL21 (DE3) expression cells. Expression cells BL21 (DE3) harboring pET-24 a(+) +ELP(A₂YA₂)_z gene or ELP(A₂YA₂)_z + M2e gene, were inoculated in 10 mL LB broth starter culture with 50µg/mL kanamycin. The starter culture was incubated at 37°C with aeration by shaking at 250 rpm overnight. Culture was centrifuged at 3500 rpm at 4°C for 15min. Pellet was resuspended in 2 mL of fresh LB media. 1L of magnificent broth medium in a 3L flask was inoculated with 500 µL of this starter culture and was incubated at 37°C, 250rpm for 24h. Cells transformed with intact pET-24 a(+) were also grown as a control for expression.

3.3. ELP purification

Cell culture was centrifuged at 10 800 *g* for 10 min in 500mL bottles and cells were harvested. Supernatant was discarded and pellet was resuspended in cold 1X PBS. Cells were lysed using French pressure cell. Cell lysate was collected in a clean pre-chilled centrifuge tube. Polyethyleneimine (PEI) 10% at a final concentration of 0.7% was added to the cell lysate to precipitate nucleic acid contaminants. Cell lysate was then centrifuged at 13 000 *g* for 10 min at 4°C. The supernatant containing soluble ELP was then transferred to a new 50 mL round bottom centrifuge tube. Inverse transition cycling (ITC) as described by McDaniel *et al.*²¹ was performed to purify the ELP: briefly, supernatant containing ELP was heated to 40°C, 2.5–5M of NaCl (sterilized) was added to the tube in a ratio of 1:1 (v:v), the tube was held at 40°C and immediately spun at 13000 *g* for 10 min at 40°C. Supernatant was

discarded and pellet was resuspended in 15mL pre-chilled 1X PBS (sterilized). To complete one cycle of ITC, 1X PBS containing resuspended pellet was then centrifuged at 20 000 g for 10 min at 4°C to remove any insoluble contaminants. Supernatant containing soluble ELP was transferred to a clean tube and pellet was discarded. Four rounds of ITC, with a minute modification of reduced spin speed of 3000 g for every hot spin after first ITC step was performed sequentially to yield purified ELP product.

3.4. ELP characterization

Purity of ELP and ELP + M2e fusion proteins was determined by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4–20% mini-PROTEAN TGX gels (Bio-Rad), which were stained with colloidal Coomassie stain. Molecular weight of purified ELP(A₂YA₂)_z and ELP(A₂YA₂)_z+ M2e fusion protein was further characterized with matrix-assisted laser desorption/ time of flight (MALDI-TOF) and MALDI-TOF/ TOF (MALDI-TOF/TOF 4800 plus mass spectrometer, Applied Biosystems). ELP sample was mixed with sinapinic acid matrix, and 1.2 µL of this mixture was spotted on a plate (384 opti-TOF 123 mm × 81mm stainless steel, Applied Biosystems). A nitrogen laser beam (355 nm) was used to ionize the co-crystallized sample. A total of 1250 shots were accumulated for each sample. Concentration of protein was measured using DC Protein assay kit (Biorad).

3.5. Inverse transition temperature of ELP

UV-visible spectrophotometer having a multicell thermoelectric temperature controller (Cary 300, Varian Instruments) was used to characterize T_i by measuring optical density (OD) of ELP at 350 nm as a function of temperature. This was done as follows: an ELP solution (25 µM) was heated from 4°C to 55°C at a rate of 1°C/min and the OD of the sample was measured. Each time prior to use, the instrument was blanked with 1X PBS with similar parameters.

3.6. Dot-blot to detect M2e antigen fused to ELP

Serial dilution of samples for ELP(A₂YA₂)_z + M2e and only ELP(A₂YA₂)_z, where Z = 24 and 36, were prepared with sterilized 1X PBS. Polyvinylidene fluoride (PVDF) membrane was pre-wetted in methanol and then in PBS containing 0.05% tween 20 (PBST). Samples were then spotted (2 µL) on a membrane and the membrane was air-dried. To reduce nonspecific binding of primary antibody, membrane was soaked in 5% nonfat dry milk in PBST for 2h at room temperature with gentle agitation, and washed three times with PBST for 5 min each. The membrane was then incubated with primary antibody (serum collected from mice immunized with M2e conjugated to gold nanoparticles from one of our previous study (35)) dissolved in 1% nonfat dry milk + PBST, using a dilution ratio of 1:1000 with gentle agitation for 1 h. To remove any unbound primary antibody, the membrane was washed four times with PBST for 5 min each. Goat anti-mouse IgG HRP secondary antibody diluted to 1:1000 in 1% nonfat dry milk + PBST was then added to the membrane and membrane was incubated at room temperature for 1 h with gentle agitation. Membrane was then washed with PBST. Chromogenic detection of the antigen–antibody complex was done by adding Pierce 1-step TMB-blotting substrate solution (Thermo Scientific, IL, USA).

Development of color was observed, and the membrane was air-dried and photographed for records (ChemiDoc™ MP Imaging system, BioRad).

3.7. Immunization of mice

BALB/c female mice aged between 6 and 8 weeks were purchased from Charles River Laboratories (MA, USA). Mice were maintained at Animal Care Services, Texas Tech University (TX, USA). All the animals were treated in accordance with protocols approved by Texas Tech University Animal Care and Use Committee (IACUC). Mice were divided into three groups with 5 mice per group. Group 1 was vaccinated with 100 µg of free M2e peptide per mouse, group 2 with 2mg ELP(A₂YA₂)₂₄ + M2e fusion protein (which is equivalent to 100µg M2e), and group 3 with 2mg ELP(A₂YA₂)₂₄ as a negative control. The mass of ELP(A₂YA₂)₂₄ + M2e equivalent to 100 µg-free M2e was calculated as follows. M2e peptide represents ~ 5% ($2723.9/50926.3 \times 100$, where 2723.9 and 50926.3 are calculated molecular weights in dalton for M2e peptide and fusion protein, respectively) of the total weight of fusion protein. Thus, 2mg ELP(A₂YA₂)₂₄ + M2e is equivalent to 100µg M2e ($0.1 \text{ mg}/0.05 = 2 \text{ mg}$). Before administration, all formulations were filter-sterilized using 0.22 µm filter membrane, and the solution was stored at -80°C until the day of immunization. On the day of immunization, prior to administration, all the doses were incubated at room temperature for 30min. For each mouse, 50µL of dose (containing the correct mass of free M2e or ELP(A₂YA₂)₂₄ + M2e or ELP(A₂YA₂)₂₄) was mixed with 50µL of Alhydrogel® 2% (InvivoGen, San Diego California) and the resulting 100 µL of formulation was intramuscularly injected using a 27 gauge hypodermic needle (Terumo Medical Corporation, MD, USA) on day 0 with a boost on day 21. Blood samples were collected through retro-orbital bleeding at days 0, 21 and 42 using a heparinized micro-hematocrit capillary tube (Fisher brand, PA, USA). Collected blood was allowed to set at room temperature and then centrifuged at 17000 g for 15min. Serum samples were collected in eppendorf tubes and stored at -20°C until further analysis.

3.8. Measurement of M2e-specific antibody response

M2e-specific immunoglobulin G (IgG) in mouse sera was determined using enzyme-linked immunosorbent assay (ELISA) as described earlier.³⁵ A 96-well plate (MaxiSorpff -Nunc, Sigma Aldrich) was coated with 50 µL of 5 µg/mL M2e peptide in PBS and incubated at 4°C overnight. 100 µL of 3% bovine serum albumin in PBS was used to block the plate and the plate was incubated for 2 h at room temperature. Plate was washed thrice with PBST using ELx405 microplate washer (BioTek, VT, USA). Serum samples were then analyzed by adding 50 µL of serum (individual or pooled) to the plate and incubating the plate at room temperature for 1 h. Plate was then washed thrice with PBST and 50 µL of goat anti-mouse IgG or anti-mouse IgG1 or anti-mouse IgG2a labeled with HRP (1:4000 dilution in PBST) was added per well. Following incubation for 1 h at room temperature, the plate was washed thrice with PBST and color was developed with OPD as substrate. After 5– 10min, 50 µL of 3% phosphoric acid was added to terminate the reaction. Absorbance at 492 nm was recorded by SpectraMax Plus384 microplate reader (Molecular Devices, LLC, CA).

3.9. Influenza virion-specific ELISA

ELISA was performed as described above for M2e-specific antibodies with the exception that the 96-well plates were coated with inactivated influenza A/WSN/1933 (H1N1). Total IgG was assessed by using the secondary goat anti-mouse IgG antibody labeled with HRP (1:4000 dilution).

4. Results

4.1. Tailoring ELP and M2e nanodomains

A schematic of the nanoscale-designed protein polymer is shown in Fig. 1. The molecule contains two domains, an ELP domain and an M2e antigen domain. M2e was attached to the N-terminal of ELP domain to preserve the natural orientation of M2e as found on influenza virions. DNA sequences for the modifier insert, ELP monomer and M2e peptide sequence used in protein synthesis are provided in Fig. 1.

Guest residue composition in the ELP sequence plays an important role in tuning the inverse transition temperature of an ELP.³⁶ We selected “alanine” and “tyrosine” as guest residues based on two major considerations. First, we wanted the ELP + M2e fusion molecule to exhibit a T_i below the physiological temperature to facilitate its purification. Since M2e is hydrophilic, we expected its conjugation to the ELP would elevate the T_i of the ELP + M2e fusion protein. Thus, to safeguard against this expected increase in T_i upon attachment of M2e, we set the targeted T_i of the ELP without M2e at approximately 25°C. Second, based on the use of synthetic polymers as vaccine adjuvants,^{33,34} it is known that hydrophobic polymer blocks can function as vaccine adjuvants. Thus, we sought to include hydrophobic amino acid domains in the ELP, and selected tyrosine. However, tyrosine by itself is expected to produce a T_i of much lower than 0°C,³⁶ thus it became important to include a less hydrophobic amino acid in the ELP construct. It is known that ELPs with only “alanine” as a guest residue exhibit T_i s of about 30°C.³⁷ Accordingly, to create a balanced ELP that has a hydrophobic domain and yet can still exhibit T_i closer to physiologic range, we selected alanine and tyrosine as the amino acids of choice in a ratio of 4:1 to construct the ELP.

4.2. Biosynthesis and molecular characterization of ELPs and ELP +M2e proteins

PRE-RDL was employed to generate a library of plasmids encoding $\text{ELP}(\text{A}_2\text{YA}_2)_z$ with $z = 3, 6, 12, 24$ and 36 . Modification of pET-24 a(+) vector allowed successful attachment of M2e antigen sequence to the N-terminus of two ELPs, $\text{ELP}(\text{A}_2\text{YA}_2)_{24}$ and $\text{ELP}(\text{A}_2\text{YA}_2)_{36}$, which was confirmed by sequencing. Recombinant plasmids harboring $\text{ELP}(\text{A}_2\text{YA}_2)_{24}$, $\text{ELP}(\text{A}_2\text{YA}_2)_{36}$, $\text{ELP}(\text{A}_2\text{YA}_2)_{24} + \text{M2e}$ and $\text{ELP}(\text{A}_2\text{YA}_2)_{36} + \text{M2e}$ were expressed in expression cells BL21 (DE3) and purified with five rounds of ITC. Expression yields, in each case, were ~ 150 mg/L of the culture after ITC. In the ELP + antigen fusion protein, M2e peptide represents ~5% [$\text{ELP}(\text{A}_2\text{YA}_2)_{24} + \text{M2e}$] and ~3.66% [$\text{ELP}(\text{A}_2\text{YA}_2)_{36} + \text{M2e}$] of the total weight of fusion protein. Accordingly, the yield of M2e was ~ 7.5 mg/L and ~ 5.5 mg/L from $\text{ELP}(\text{A}_2\text{YA}_2)_{24} + \text{M2e}$ and $\text{ELP}(\text{A}_2\text{YA}_2)_{36} + \text{M2e}$, respectively.

To confirm the purity of ELPs and to verify their molecular weights, sodium dodecyl sulfate poly-acrylamide gel electrophoresis and MALDI-TOF was performed on the purified ELPs. Each sample ran as a single band (Fig. 2) indicating purity of preparation. Appearance of the bands at a higher molecular weight than their actual molecular weights when compared to standard bands is consistent with earlier observation that ELPs tend to migrate ~ 20% higher than their absolute molecular mass.³⁸ In each case bands representing ELP + M2e fusion proteins ran slightly higher than their “only ELP” counterparts due to the additional load of ~ 2.6 kDa of M2e peptide carried by the fusion protein (Fig. 2). MALDI-TOF analysis of ELPs with and without M2e further revealed the purity of the sample (Table 1). The observed (MALDI) molecular weights of the ELPs were similar to their respective calculated molecular weights. As expected, the spectra for ELP + M2e fusion proteins was approximately 2.6 kDa heavier than their free ELP counterparts, further confirming successful expression of ELP + M2e fusion proteins.

4.3. Thermal characterization of ELPs and ELP + M2e proteins

After successful expression and purification of ELPs and ELP + M2e fusion proteins we sought to investigate their T_i s. Figure 3 shows turbidity profiles of ELP solutions at 25 μ M, as a function of temperature. A sharp increase in OD is seen at the T_i s of the respective ELPs and their fusion proteins. It can be seen that ELP(A₂YA₂)₃₆ has the lowest T_i (25°C) followed by ELP(A₂YA₂)₃₆ + M2e (28°C), ELP(A₂YA₂)₂₄ (32°C) and ELP(A₂YA₂)₂₄ + M2e (36°C). A decrease in T_i was observed with increase in molecular weight of both ELP and ELP + M2e fusion proteins. This trend is in agreement with previous studies in which increase in the molecular weight of ELPs caused a depression in their transition temperature at a given concentration.³⁹ Incorporation of hydrophilic M2e sequence elevated the T_i for the fusion proteins as compared to their “only ELP” counterparts. Interestingly, for both ELP(A₂YA₂)₂₄ and ELP(A₂YA₂)₃₆, the increase in T_i of ELP fusion protein was ~ 12% compared to the respective “only ELP” counterparts. These observations are of particular importance to our future studies where we would like to analyze the immunogenic properties of different antigens keeping the ELP base the same. Aggregation of the ELPs was completely reversible and the solution became clear upon decreasing the temperature below T_i (data not shown).

In a study done by Christensen *et al.*,⁴⁰ it has been seen that the fusion of hydrophilic proteins to an ELP can elevate the transition temperature of the resulting ELP + protein fusion molecule as compared to their “only ELP” counterpart. They found that the elevation in T_i was primarily due to the presence of amino acids — glutamic acid (E), aspartic acid (D), lysine (K) and arginine (R) at the protein surface. The characteristic transition temperature for amino acids E, D and K is relatively high as compared to other amino acids.⁴¹ In M2e, amino acids E and D constitute approximately 21%, while the amino acids E, D and R constitute approximately 29% of the total amino acids. The presence of these charged amino acids along with other neutral polar amino acids such as asparagine (N), serine (S) and threonine (T) might explain the elevation of the transition temperature of ELP + M2e fusion protein as compared to “only ELP” counterparts.

4.4. Dot-blot assay for in vitro immunogenicity assessment of ELP + M2e

For ELP + M2e fusion protein to be useful as a vaccine antigen it is important that M2e in the fusion protein retains its antigenic nature. Therefore, we next investigated the antigenicity of M2e in the ELP + M2e fusion protein through its interaction with M2e-specific antibody. We have previously used gold nanoparticles conjugated with synthetic M2e peptides as a novel universal influenza vaccine.³⁵ Serum from mice immunized with these gold nanoparticle-M2e conjugates was used as primary antibodies in a dot-blot test. As seen in Fig. 4(a) and 4(b), M2e in the fusion proteins ELP(A₂YA₂)₂₄ + M2e and ELP(A₂YA₂)₃₆ + M2e, respectively can be recognized by the anti-M2e antibodies. No dots were seen for spots blotted with only ELP protein at the same concentration, indicating that only the M2e peptide on the ELP is recognizable by the antibodies. Further, no dots were seen for only M2e peptide-blots because the peptide is small and it gets easily washed away during the membrane wash steps. However, the immune serum used for dot-blot does indeed bind M2e peptide, and this has been demonstrated via ELISA in our earlier publication.³⁵

4.5. Immune response to EL(A₂YA₂)+M2e antigen proteins

Having confirmed the *in vitro* antigenicity of M2e in ELP + M2e fusion proteins through the dot-blot test, we next assessed their potential to generate an immune response *in vivo*. M2e as free peptide and as ELP(A₂YA₂)₂₄ + M2e fusion peptide were used to immunize mice. M2e dose was fixed at 100 µg. ELP(A₂YA₂)₂₄ without M2e was used as a negative control. Alum is an FDA-approved adjuvant, and we included it in the form of Alhydrogel® in the vaccine formulation to maximize the immune response. Antibody titration curve for day 21 pooled sera [Fig. 5(a)] shows that after a single dose ELP(A₂YA₂)₂₄ + M2e can lead to increase in serum anti-M2e IgG, however, M2e peptide and ELP(A₂YA₂)₂₄ show no appreciable increase. After a second dose given on day 21, serum analysis at day 42 shows that ELP(A₂YA₂)₂₄ + M2e demonstrated a significant increase in serum anti-M2e IgG, while the IgG increase in M2e peptide group was only marginal [Fig. 5(b)]. M2e-specific antibody remained undetectable in mice immunized with ELP(A₂YA₂)₂₄. To further assess inter-animal variability to immunizations, a serum dilution of 1:800 was selected to obtain relative amounts of anti-M2e IgGs [Fig. 5(c)]. At day 42, M2e fused to ELP(A₂YA₂)₂₄ induced significantly higher anti-M2e IgG compared to free M2e and ELP(A₂YA₂)₂₄ ($p < 0.003$). Our laboratory is currently performing follow-up experiments to compare how different repeats of ELP might affect the immune response.

Recently, ELPs have been applied for synthesis of vaccine antigens in plants. A study by Phan *et al.*²⁸ has demonstrated that ELP-HA fusion protein can be produced in tobacco plants, and upon HA trimerization, the antigen can induce neutralizing antibodies against homologous and heterologous avian influenza viruses. In another study, Phan *et al.*²⁷ produced both ELP-HA and -NA fusion proteins in tobacco plants, and demonstrated their *in vitro* antigenic properties. Apart from influenza, ELP technology has also been extended to tuberculosis vaccine. García-Arevalo *et al.*⁴² have developed amphiphilic ELP-tuberculosis antigen fusion proteins that can self-assemble into nanoparticles displaying multiple copies of the antigen on their surface. In our current study, we have investigated the ability of a hydrophobic ELP sequence to enhance the immunogenicity of M2e, which by itself is a weak influenza antigen. We have used a bacterial expression system over plants

because M2e is a small peptide, and it does not possess a secondary structure or require post-translational modifications that may require use of a plant cellular machinery. Furthermore, bacterial expression is faster and more convenient as compared to a plant-based protein expression system, for example, unlike plants, the bacterial expression system does not require additional extraction steps, such as leaf homogenization to obtain ELPs.

We observed that ELP + M2e fusion protein does indeed demonstrate significantly enhanced M2e immunogenicity compared to free M2e peptide. This observation is consistent with previous studies wherein M2e has been conjugated to carrier proteins such as bovine serum albumin,¹⁰ keyhole limpet hemocyanin¹⁴ and glutathione S-transferase.⁴³ However, ELP + M2e fundamentally differs from these previous M2e-carrier constructs because ELP + M2e fusion protein contains only one copy of the epitope per molecule, as opposed to multiple M2e peptides that are attached to the carrier proteins. Thus, there exists the possibility of further enhancing the immunogenicity of M2e by creating self-assembling nanoparticles using amphiphilic ELPs that can display multiple copies of M2e on their surface.

It is important, to note that while we added alum as an adjuvant, the immunogenicity of ELP(A₂YA₂)₂₄ + M2e, may very well be enhanced due to its aggregated state at physiological temperature. However, additional *in vivo* experiments that use ELP(A₂YA₂)₂₄ + M2e alone as a vaccine at different concentrations will be needed to further understand the contribution of ELP(A₂YA₂)₂₄ + M2e aggregation towards enhancement in immune response.

4.6. Serum IgG subtypes

We examined Th1 versus Th2 response induced by the ELP(A₂YA₂)₂₄ + M2e protein by assessing M2e-specific serum IgG1 and IgG2a antibodies. At day 42 post-immunization, the ELP(A₂YA₂)₂₄ + M2e protein induced higher IgG1 and IgG2a antibody levels compared to M2e and ELP(A₂YA₂)₂₄ alone (Fig. 6). Further, for ELP(A₂YA₂)₂₄ + M2e group the IgG1 amount was higher as compared to IgG2a. It has generally been accepted that Th1 type immune response is more critical for restricting viral infections.^{44,45} In other words, higher levels of M2e-specific IgG2a antibodies have a better correlate with viral-clearance and protection against influenza challenge. Previous studies have shown that CpG (Cytosine Guanine rich single stranded oligonucleotide) motifs induce higher levels of antigen-specific IgG2a, i.e., Th1 humoral and cell-mediated response.⁴⁶⁻⁴⁹ Indeed, in a recent study it was found that addition of soluble CpG to a formulation based on gold-nanoparticle-M2e conjugate not only improved the M2e-specific IgG1 antibody response, but also significantly enhanced M2e-specific IgG2a antibody response, resulting in an elevated yet balanced IgG1/IgG2a response.³⁵ Accordingly, to enhance IgG2a response, in future studies we intend to investigate the ability of CpG as an adjuvant.

4.7. In vitro reactivity of antibodies to influenza virus

We further assessed the ability of serum IgG to bind to native M2 expressed on the surface of influenza virus by coating WSN-H1N1 as ELISA capture antigen. It can be seen from Fig. 7 that immunization with ELP(A₂YA₂)₂₄ or M2e does not induce any significant virus-binding IgG, however, immunization with ELP(A₂YA₂)₂₄ + M2e induces significant IgG-

binding to WSN-H1N1 ($p < 0.05$). Overall, this suggests that the antibodies generated have potential to bind native M2, and thus may protect mice from a lethal challenge with WSN-H1N1. This hypothesis will be tested in our laboratory in future, and will additionally include improved ELP-M2e constructs as antigens.

5. Conclusion

In this study, we synthesized nanoscale-designed ELP + M2e proteins and characterized their immunogenic properties. Four alanine and one tyrosine substitutions for “X” in “GXGVP” were made to create the ELP repeat sequence — A₂YA₂. Through Pre-RDL approach, ELP(A₂YA₂)₂₄ and ELP(A₂YA₂)₃₆, both with and without fusion of M2e were synthesized and purified. The two ELPs without M2e exhibited T_i below physiological range. Although fusion of M2e peptide elevated the transition temperature of both ELPs by approximately 12%, yet it still remained below the physiological temperature. A dot-blot test confirmed the ability of M2e in ELP + M2e fusion proteins to be recognized by M2e-specific antibodies. Immunization of mice with ELP(A₂YA₂)₂₄ + M2e along with alum as an adjuvant generated significantly higher serum M2e-specific antibodies in mice compared to mice receiving M2e peptide alone with alum. Injection of ELP(A₂YA₂)₂₄ with alum, did not induce any detectable serum anti-M2e antibodies in mice. The serum antibodies could also bind to native M2 expressed on WSN-H1N1. Overall, this study presents the proof-of-concept of using ELP + M2e proteins to enhance immunogenicity of M2e, which could help to design a universal influenza vaccine.

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ELP domain : (GAGVPGAGVPGYGVPGAGVPGAGVP)_{24 or 36}

ELP (A₂YA₂)₂₄ or 36

ELP+M2e nanodomained molecule:

M2e

ELP (A₂YA₂)₂₄ or 36

Antigen domain

GENE SEQUENCES USED

pET-24 a(+)-modifier insert sequence

5'	C	T	A	G	A	A	A	T	A	A	T	T	T	G	T	T	A	A	C	T	T	A	A	G	A	A	G	G	A	G	G	A	G	T	A	C	A	T	A	T	G	G	G	C	T	A	C	T	G	A	T	A	A	T	G	A	T	C	T	T	C	A	G	3'				
3'									T	T	A	T	T	A	A	A	C	A	A	T	T	G	A	A	T	T	C	T	T	C	C	T	C	A	T	G	T	A	T	A	C	C	C	G	A	T	G	A	C	T	A	T	T	A	C	T	A	G	A	A	G	T	C	C	T	A	G	5'
XBAI overhang																																								BAMHI overhang																												

ELP monomer sequence

	G	A	G	V	P	G	A	G	V	P	G	Y	G	V	P	G	A	G	V	P	G	A	G	V	P	G																	
5'	T	G	C	A	G	G	C	T	A	C	G	G	G	T	G	C	T	G	G	C	G	G	G	T	T	A	C	G	G	T	T	C	C	A	G	G	3'						
3'	C	C	A	C	G	T	C	C	G	C	A	T	G	G	C	C	A	A	G	G	T	C	C	G	C	G	T	C	C	A	C	A	T	G	G	C	C	A	A	G	G	T	5'

M2e sequence

	M	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	S	R	S	N	D	S	S	D																																															
5'	T	A	T	G	T	C	T	G	C	T	G	A	C	G	A	A	G	T	A	G	A	A	C	T	C	G	A	T	T	C	G	A	A	C	G	A	A	T	G	G	G	G	C	T	C	C	C	G	T	A	G	C	A	A	C	G	A	T	T	C	C	A	G	C	G	A	C	G	G		3'
3'		A	C	A	G	A	G	A	C	G	A	C	T	T	C	A	T	C	T	T	T	G	A	G	G	C	T	A	A	G	C	G	T	T	G	C	T	A	C	C	C	G	A	G	G	G	C	A	T	C	G	T	T	G	C	T	A	A	G	G	T	C	G	C	T	G		5'			

Fig. 1. Schematic of M2e-ELP nanoscale-designed protein polymers and gene sequences used for their synthesis.

Fig. 2. SDS-PAGE analysis of expressed ELPs. Lane A represents a molecular weight standard with size indicated on the left in kilodalton (kDa). Lanes B, C, D and E represent expressed ELP(A₂YA₂)₂₄, ELP(A₂YA₂)₂₄ + M2e, ELP(A₂YA₂)₃₆ and ELP(A₂YA₂)₃₆ + M2e, respectively, with the calculated molecular weight of each purified protein in kDa given at the bottom.

Fig. 3.

Transition temperature for expressed ELPs. Transition temperature was characterized by measuring turbidity (OD at 350 nm — OD₃₅₀) of 25 μM of free ELP as well as ELP-M2e proteins as function of temperature. Transition temperature of free ELP is depressed with increase in molecular weight but is elevated by fusion of hydrophilic M2e sequence.

Fig. 4.

Dot-blot assay of ELP + M2e protein. Dot-blot assay was performed to study antigen recognition on (a) ELP(A_2YA_2)₂₄ + M2e and (b) ELP(A_2YA_2)₃₆ + M2e. Different concentrations of ELP + M2e fusion proteins, free M2e peptide and ELP without M2e were spotted. The concentrations for each spot are mentioned at the top. Color development revealing M2e recognition on ELP + M2e fusion protein can be seen in the top-most rows for both (a) and (b). No color development was observed for the middle and bottom rows where free M2e peptide and ELP without M2e were spotted for both (a) and (b), demonstrating that M2e in ELP + M2e is specifically recognized by anti-M2e antibodies, which do not cross react with ELP (color online).

Fig. 5. M2e-specific antibody response in mice upon vaccination with ELP(A₂YA₂)₂₄ + M2e. Groups of mice ($n = 5$ per group) were vaccinated on days 0 and 21 and serum was collected on days 0, 21 and 42 for analysis using ELISA to determine anti-M2e IgG in serum. (a) Titration curve of pooled day 21 mouse serum demonstrating anti-M2e IgG stimulation with ELP(A₂YA₂)₂₄ + M2e but not free M2e peptide and ELP(A₂YA₂)₂₄. (b) Titration curve of pooled day 42 mouse serum demonstrating strong anti-M2e IgG stimulation with ELP(A₂YA₂)₂₄ + M2e, weak stimulation with free M2e peptide, and none with ELP(A₂YA₂)₂₄. (c) Anti-M2e IgG in individual mice serum at each time point (days 0, 21 and 42) at a dilution 1:800. Day 42 anti-M2e IgG response by ELP(A₂YA₂)₂₄ + M2e group is significantly higher compared to all groups at day 42 ($p < 0.003$), and also compared to other days (day 0: $p < 0.0014$; day 21: $p < 0.007$). Each symbol represents a mouse, the error bar represents standard deviation, and the horizontal bar represents the mean.

Fig. 6. M2e-specific IgG subtypes in mouse serum. Groups of mice ($n = 5$ per group) were vaccinated on days 0 and 21. Mouse serum from days 0 and 42 was diluted to 1:400 and assessed using ELISA to determine anti-M2e IgG1 and IgG2a. (a) M2e-specific IgG1, and (b) M2e-specific IgG2a. OD measured at 492 nm wavelength. mean $\pm$ SEM.

Fig. 7.

Reactivity of mouse serum-IgG (1:20 dilution) against native M2 expressed on influenza A virus WSN-H1N1. Groups of mice ($n = 5$ per group) were vaccinated on days 0 and 21. Mouse serum from day 42 was diluted to 1:20 and assessed using ELISA to determine IgG against WSN-H1N1. OD measured at 492 nm wavelength. mean $\pm$ SEM.

Table 1

Calculated and observed molecular weights of ELPs.

ELP	Mass (Da)		
	Calculated	Observed (MALDI)	% Difference
(A ₂ YA ₂) ₂₄	48 351	48 236	0.24
(A ₂ YA ₂) ₂₄ ⁺ M2e	50 926	50 787	0.27
(A ₂ YA ₂) ₃₆	72 342	72 134	0.28
(A ₂ YA ₂) ₃₆ ⁺ M2e	74 917	74 737	0.24