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Research paper

Altered antigenicity and immunogenicity of human papillomavirus virus-like particles in the presence of thimerosal

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ABSTRACT

Thimerosal has been widely used as a preservative in human vaccines for decades. Thimerosal, a thiol capping agent with ethyl mercury being the active degradant, could have impacts on the vaccine potency due to potential thiol modification. The effects on the antigenicity and immunogenicity of human papillomavirus (HPV) virus-like particles (VLPs) in the presence of thimerosal was studied. In general, reduced binding activity was observed between HPV antigens and monoclonal antibodies (mAbs) upon thimerosal treatment, accompanied by reduced protein conformational stability. The immunogenicity of a pentavalent vaccine formulation (HPV6, HPV11, HPV16, HPV18 and hepatitis E virus) with or without thimerosal was studied in mice. The functional antibody titres, as well as the binding titres, were determined, showing a substantial decrease for vaccine formulations containing thimerosal for HPV16/18. Similarly, epitope-specific competition assays using specific and functional mAbs as tracers also showed a significant reduction in immunogenicity for HPV16/18 in the presence of thimerosal. Structural alterations in the capsid protein for HPV18 were observed with cryo-electron microscopy and 3-dimensional reconstruction in the comparative structural analysis. The results should alert scientists in formulation development field on the choice for vaccine preservatives, in particular for thiol-containing antigens.

1. Introduction

Thimerosal is an organic mercury-containing compound that can inhibit bacterial and fungal growth. It was introduced in the 1930s and has been the most commonly used vaccine preservative since the 1930s [1–3]. Although there is no direct evidence to prove the health risk of thimerosal in vaccines, the use of thimerosal-containing vaccines has declined significantly since 1999 [4–7]. Currently, thimerosal is no longer used in single-dose vials or in paediatric vaccines. However, the addition of a preservative in multi-dose vials is still a common practice for some vaccines, such as multi-dose vaccines [8–10].

Thimerosal is viewed as a thiol 'capping' agent. It hydrolyses spontaneously to thiosalicylic acid and ethyl mercury in an aqueous solution. Ethyl mercury can interact with the available thiol group of cysteine (Cys) in the protein solution (Fig. 1A) [11,12]. As a result, the enzyme activity of bacteria is inhibited by thimerosal because of such modifications [13]. Similarly, the protein in the vaccine could be adversely affected by the thiol modification [11]. In response to such a concern, other alternative preservatives, such as 2-phenoxyethanol (2-PE), have been developed to replace thimerosal in multi-dose vaccines over the last two decades [1,14].

To study the effect of thimerosal on thiol-containing immunogens, HEV p239 and four types of HPV VLPs were used in this experiment. HEV causes epidemic hepatitis and its first prophylactic vaccine (Hecolin, Xiamen Innovax Biotech Co. Ltd) was licensed in 2012 in China [15,16]. HPV is the primary cause of genital warts and cervical cancer. The first HPV tetravalent vaccine (HPV6/11/16/18, Gardasil, Merck and Co., Inc.) was licensed in 2006 with the upgraded version of Gardasil-9 licensed in 2014 [17–19]. HPV vaccines are being adopted globally for different age groups across many countries.

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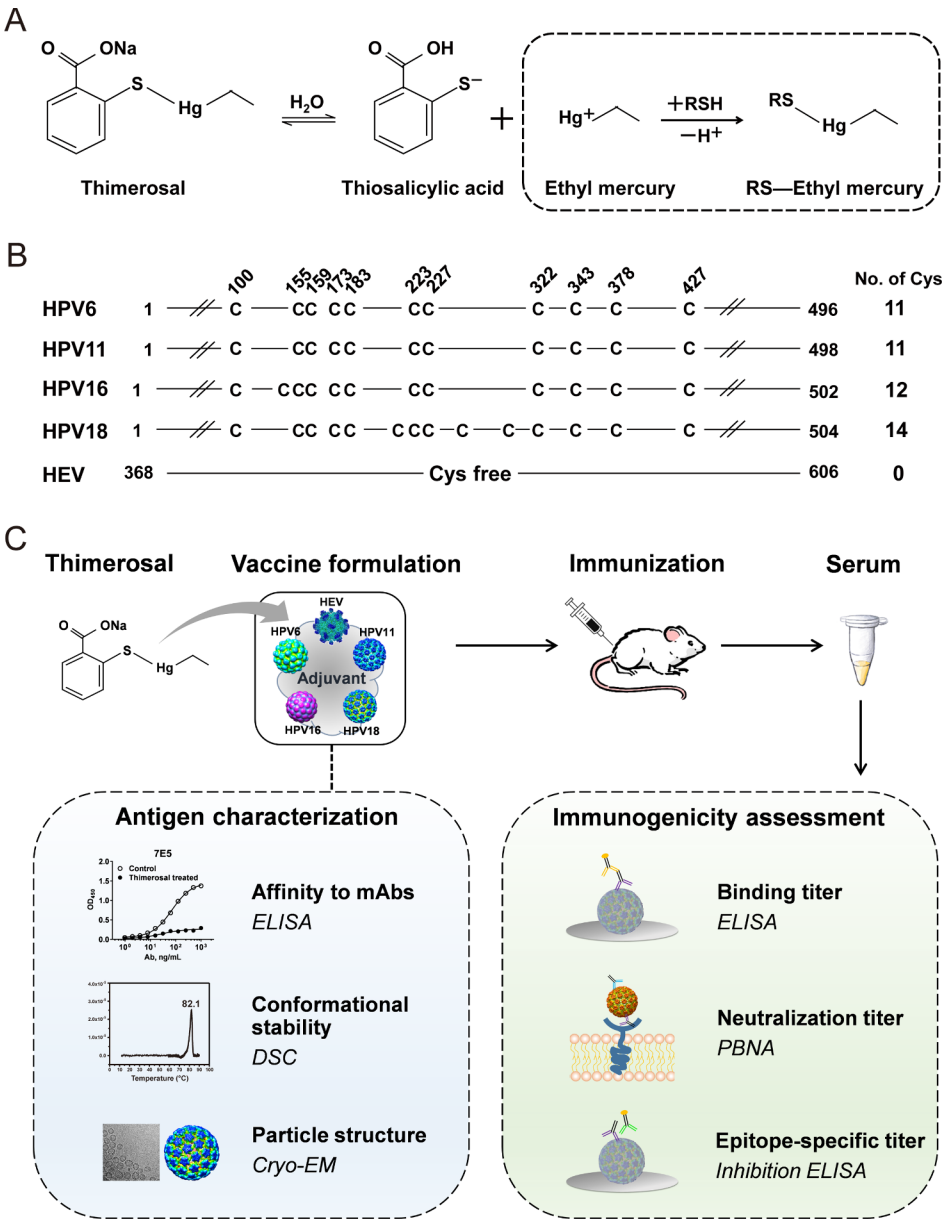

Fig. 1. Alignment of Cys residues on HPV L1 proteins, the thimerosal hydrolysis process, an illustration of the experimental procedures used for an antigenicity and immunogenicity assessment after thimerosal treatment. (A) The reaction process of thimerosal and the free thiol groups in an aqueous solution is shown. Thimerosal hydrolyses into thiosalicylic acid and ethyl mercury in aqueous solutions. The latter binds to free thiol in aqueous solution. (B) The total number of Cys residues contained in different types of HPV L1 proteins. The top numbers represent the position of the Cys residues in the HPV18 L1 protein, starting from the N-terminus, and the HEV p239 protein is Cys-free. (C) The antigenicity and immunogenicity of antigens with and without thimerosal treatment is shown. Different concentrations of thimerosal were added into the pentavalent vaccine formulation (HPV6, HPV11, HPV16, HPV18 and HEV). The properties of thimerosal-treated antigens were tested by ELISA, DSC and cryo-EM. The mice were immunized with formulations that contained different amounts of thimerosal via *i.m.*, and the serum samples were collected weekly. The immunogenicity was measured by a direct-binding ELISA, an epitope-specific inhibition ELISA and pseudovirus-based neutralization assay (PBNA).

In this work, the specific effects of thimerosal on the antigenicity and immunogenicity of five antigens were evaluated both *in vitro* and *in vivo* in a quantitative manner. By assaying the serological response, the total antibody levels and functional antibody levels in mouse serum were quantitated. For the antigen showing the highest sensitivity to thimerosal, structural alterations were observed on HPV18 VLP in the presence of thimerosal with cryo-EM 3D reconstruction. Such an observation justifies a detailed characterization of the molecular structure, antigenicity and immunogenicity during vaccine development when thiol-containing antigens are being used.

2. Materials and methods

2.1. Recombinant HPV and HEV VLPs

The pentavalent vaccine (HPV6, HPV11, HPV16, HPV18 and HEV) was produced by Xiamen Innovax Biotech Co. Ltd. (Xiamen, China). The VLPs of HPV6, HPV11, HPV16, HPV18 and HEV were expressed in *Escherichia coli*, and the production and purification of these recombinant VLPs have been described previously [20–24].

2.2. Vaccine formulation

Thimerosal (merthiolate sodium) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). An aluminium-based adjuvant was prepared in-house, which was a suspension composed of aluminium hydroxyphosphate [25]. Considering the different median effective doses for each antigen, different doses were used for each antigen. Accordingly, each antigen was diluted to the appropriate concentration using the aluminium-based adjuvant in the final formulation (HPV6 0.9 μg/mL, HPV11 0.9 μg/mL, HPV16 1.2 μg/mL, HPV18 0.6 μg/mL, an HEV 0.9 μg/mL). The Al content in the final formulation was 42 μg per dose (100 μL per mouse). Different amounts of thimerosal, i.e., 0%, 0.002%, 0.01% and 0.05% (weight/volume), were added into the final vaccine formulation for mouse immunization. The vaccine formulations were stored at 4 °C throughout. The first

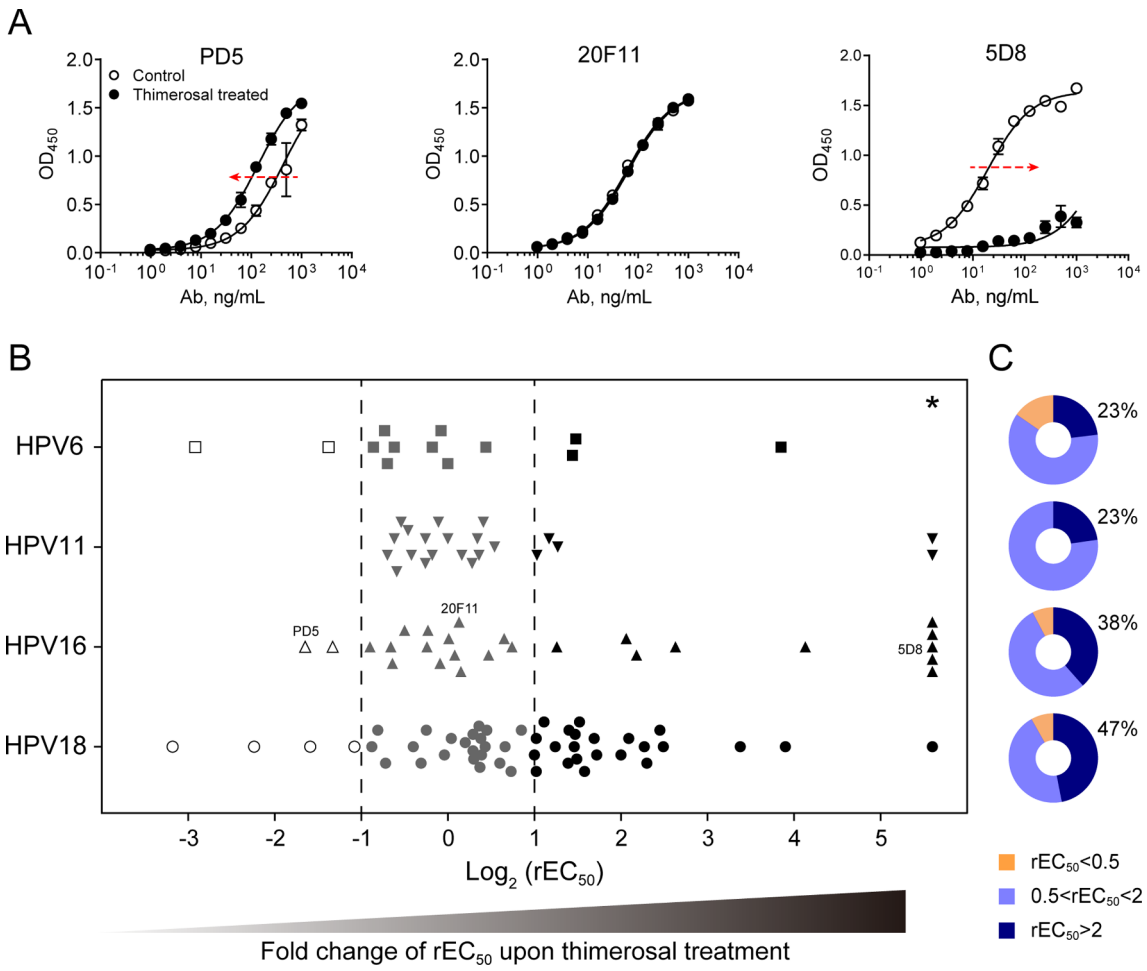

Fig. 2. Quantitative assessment of the impact on the antigenicity of four HPV antigens by thimerosal treatment. The changes in the relative binding activities of mAbs to antigens as a result of thimerosal treatment is reflected quantitatively by the fold changes in the EC_{50} values. (A) Representative binding profiles of three anti-HPV16 mAbs (PD5, 20F11 and 5D8) with distinctively different binding profiles to thimerosal-treated VLP compared to that of the control as follows: preferring thimerosal-treated VLP (PD5), insensitive for thimerosal-treated VLP (20F11) and sensitive for thimerosal-treated VLP (5D8). The red arrows indicate the direction of the change in binding ability. (B) The distribution of mAbs based on their relative binding abilities to the HPV6, 11, 16, 18 antigens after thimerosal treatment. The mAbs in the antibody panel were used to bind to normal VLPs and to thimerosal-treated VLPs. Relative EC_{50} = EC_{50} (thimerosal-treated)/EC_{50} (control). The weaker the binding ability of the mAbs to the thimerosal-treated VLPs, the larger the rEC_{50} value shown. The dots marked * represent the mAbs that are hardly binding to the thimerosal-treated VLPs, and the EC_{50} values of these mAbs could not be calculated. (C) The percentage of no. of mAbs for each category in the whole mAb panel is shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

injection was made one week from vaccine preparation.

2.3. Monoclonal antibodies (mAbs)

Murine monoclonal antibodies were generated in-house by standard hybridoma technology. Immunoglobulin G antibodies were purified from mouse ascites fluid using a protein A column (Shanghai Hua Mei Experiment Instrument Plant, Shanghai, China). A human monoclonal anti-HPV16 IgG mAb (26D1) was generated and purified as previously reported [26]. When necessary, the purified mAbs were labelled with horse radish peroxidase (HRP) through a standard sodium periodate conjugation method [27].

2.4. Animals and immunization schedule

Six-week-old female BALB/c mice were randomly divided into four groups (n = 10/group). All mice were immunized three times at 0, 2, and 4 weeks via the intramuscular route (i.m.) with 100 µL of the abovementioned formulations. The serum samples were collected weekly starting at the time of the first immunization. The pre-immunization serum samples were also collected as assay controls. The last serum samples were collected at 10 weeks before the mice were euthanized. All animal experiments with the mice were approved by the Medical Ethics Committee of the School of Public Health at Xiamen University. All procedures were conducted in accordance with the EU Directive 2010/63/EU for animal experiments to minimize the suffering during vaccination, blood collection, and euthanasia.

2.5. Direct binding ELISA

The 96-well plates were coated with 100 ng/well of normal or thimerosal-treated recombinant protein antigens in 50 mM phosphate buffer (pH 7.4) overnight at 4 °C. After blocking and washing, the mAbs were serially diluted by 2-fold starting at a concentration of 1.0 µg/mL, and the plates were incubated for 1 h at 25 °C. After washing, a goat anti-mouse IgG-HRP antibody was diluted in assay diluent (1:5000) and was added into the plates following an incubation at 25 °C for 1 h. After washing, 100 µL/well of tetramethylbenzidine substrate solution was added. The plates were incubated for 10 min at 25 °C, and the reaction was quenched with 50 µL/well of 2 M sulfuric acid. The optical density (OD) at 450 nm (630 nm reference) was measured by a microplate reader. Duplicate wells on the same plate for each dilution were

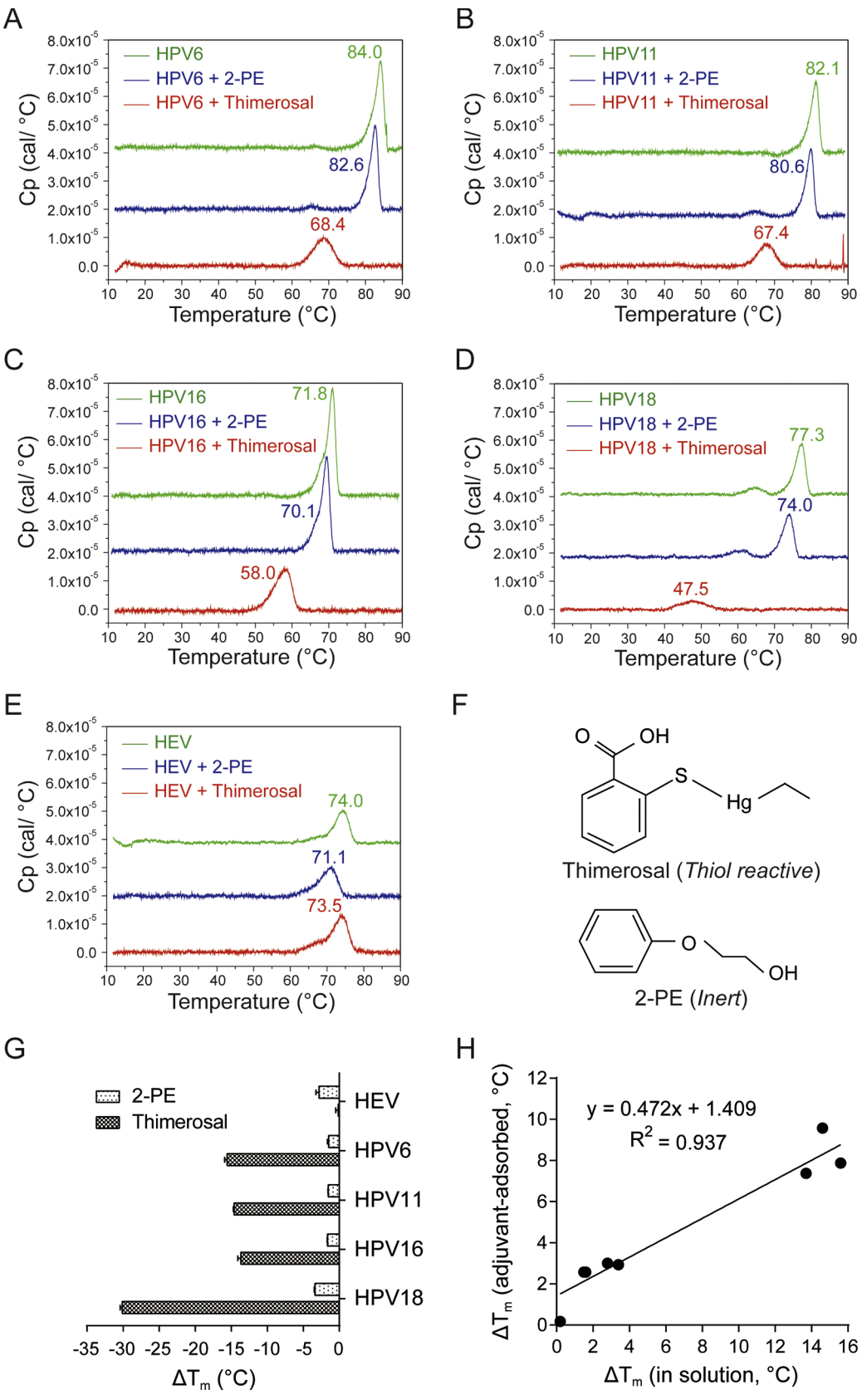

Fig. 3. Protein conformational stability measurement in the presence of two different preservatives, i.e., thimerosal and 2-PE. (A-E) The DSC profiles of five different antigens (HPV6, HPV11, HPV16, HPV18 VLP and HEV p239) in the presence of thimerosal or 2-PE. The trace coloured in green represents normal VLP, the traces coloured in blue and in red represent 2-PE-treated and thimerosal-treated VLPs, respectively. (F) The molecular structures of thimerosal and 2-PE are shown. (G) The changes in the protein conformational stability are reflected by the ΔT_m values between the normal and thimerosal or 2-PE treated antigens. Three independent measurements were performed for each sample, and the values are presented as the mean ± s.d.. (H) A scatter plot of the correlation between ΔT_m (adjuvant-adsorbed) and ΔT_m (in solution) shows similar patterns in enthalpy changes, regardless of being in solution or adsorbed onto a particulate adjuvant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

measured for all binding analyses. The median effective concentration (EC_{50}) for a given mAb was calculated with GraphPad Prism (version 7.0, GraphPad Software, Inc., La Jolla, CA, USA) from a 4-parameter logistic fit based on the averaged OD values versus the mAb dilutions. The fold change of the binding activity upon thimerosal treatment was determined by the relative EC_{50} (rEC_{50}), which was calculated based on the ratio of EC_{50} (sample)/EC_{50} (control).

Table 1
The transition temperature (T_m) for the antigens in solution in the presence of preservatives, i.e., 2-PE or thimerosal (thi). The T_m values from three independent measurements and the averaged values are reported.

Antigen	Treatment	T_m (°C)					ΔT_m (°C)[a]
		T_m-1	T_m-2	T_m-3	Average	RSD	
HEV p239	Control	74.0	74.0	73.7	73.9	0.2%	
	+0.5% 2-PE	71.1	70.8	71.3	71.1	0.4%	−2.8
	+0.05% Thi	73.5	73.8	73.9	73.7	0.3%	−0.2
HPV6 VLP	Control	84.0	84.0	84.1	84.0	0.1%	
	+0.5% 2-PE	82.6	82.6	82.4	82.5	0.1%	−1.5
	+0.05% Thi	68.4	68.8	68.2	68.5	0.4%	−15.6
HPV11 VLP	Control	82.1	82.1	82.1	82.1	0.0%	
	+0.5% 2-PE	80.6	80.6	80.5	80.6	0.1%	−1.5
	+0.05% Thi	67.4	67.5	67.6	67.5	0.1%	−14.6
HPV16 VLP	Control	71.8	71.9	71.8	71.8	0.1%	
	+0.5% 2-PE	70.1	70.3	70.2	70.2	0.1%	−1.6
	+0.05% Thi	58.0	58.7	57.8	58.2	0.8%	−13.7
HPV18 VLP	Control	77.3	77.2	77.3	77.3	0.1%	
	+0.5% 2-PE	74.0	73.7	73.9	73.9	0.2%	−3.4
	+0.05% Thi	47.5	46.9	47.0	47.1	0.7%	−30.1

[a] ΔT_m is the difference in the T_m average values for the treated and control samples from the DSC determination.

2.6. Binding titre determination in serum samples

Mouse serum samples were serially diluted by 3-fold starting at 1:100 on the antigen-coated plates. The plates were then incubated for 1 h at 25 °C. After the plates were washed, 100 µL/well of a 1:5000 diluted goat anti-mouse IgG-HRP antibody was added to the plates. The plates were then incubated for 1 h at 25 °C. The chromogenic reaction and OD_{450} detection methods are the same as the procedures described in Section 2.5. Duplicate wells on the same plate for each dilution were measured for all the serum samples. The cut-off value was calculated as the mean of the control OD_{450} value plus three times the standard deviation (s. d.) of the pre-immunization serum samples (n = 40). The binding titre is defined as the first dilution with a value larger than the cut-off value.

2.7. Inhibition ELISA for epitope specific titres

Mouse serum samples (1:100 diluted) were added to the plates in a volume of 100 µL per well. The plates were incubated for 0.5 h at 25 °C. Then, 100 µL of the diluted HRP-conjugated detection mAb was added, followed by a 1 h incubation at 25 °C. After colour development, the OD_{450} was measured. All samples were detected in duplicate on the same plate. The reported value was the average of the two repeats. The control was the well that contained only the mAb-HRP without the competing serum samples. The inhibition rate was calculated as [OD_{450} (control well) − OD_{450} (inhibited well)]/OD_{450} (control well) × 100%.

2.8. Pseudovirus-based neutralization assay (PBNA)

First, HPV pseudovirus were expressed by co-transfecting 293FT cells as previously described [28]. Then, fresh 293FT cells were plated in the 96-well cell culture plate at a density of 1.5×10^4 cells/well in 100 µL medium for at least 4 h in the cell incubator. Meanwhile, 120 µL of diluted serum samples (1:100) were added into the first well of a 96-well dilution plate followed by a 2-fold serial dilution. Then, 60 µL of diluted pseudovirus (2×10^5 $TCID_{50}$/µL) was added into the dilution plate. After incubating for 30 min, 100 µL of the serum- pseudovirus mixture was transferred to the cell culture plates. The cell culture plates were incubated for 72 h in a cell incubator. The number of pseudovirus-infected green fluorescent protein expressing spots was counted using an ImmunoSpot device (Cellular Technology Ltd, Cleveland, OH). The serum half neutralization titre (NT_{50}) was defined as the maximum dilution of serum with a 50% inhibition of pseudovirus infection based on the number of spots. Each sample was measured in duplicate in one cell plate, and the value represents the average of two repeat NT_{50} values. The negative control was a well that contained only the pseudovirus. The blank control was the well that contained only the medium.

2.9. Differential scanning calorimetry (DSC)

DSC was performed using a MicroCal VP-DSC instrument (GE Healthcare MicroCal Products Group, Northampton, MA, USA). All samples were diluted to a final concentration of 200 µg/mL in the presence of 0.05% thimerosal (weight/volume) or 0.5% 2-PE (volume/volume) for at least 48 h (stored at 4 °C) prior to the DSC measurement. Then, the samples were heated with a programmed temperature ramping rate of 1 °C/min, and the temperature ranged from 10 °C to 90 °C. The transition midpoint temperature (T_m) values were calculated by MicroCal Origin (version 7.0, Origin-Lab Corporation, Northampton, MA) based on the melting curves. Three independent runs were performed on each sample, yielding three independent T_m values.

2.10. Cryo-EM and the 3D reconstruction of HPV18 VLPs

The HPV18 VLPs were treated with 0.05% thimerosal (weight/volume) at 4 °C for 48 h. Then, the control or thimerosal-treated HPV18 VLPs were vitrified on Quantifoil holey carbon grids in a FEI Vitrobot (FEI, Thermo Fisher Scientific, Waltham, MA). The images were recorded with an FEI Falcon II direct detector camera in an FEI TF30 microscope at 300 kV with an electron dose of ~25 e/Å^2. A total of ~900 particle images from 831 micrographs for each sample were manually boxed and then extracted with the program EMAN2 [29]. The RELION and AUTO3DEM program suites were used for image processing and 3D reconstruction [30,31].

2.11. Statistical analysis

GraphPad Prism (version 7.0, GraphPad Software, Inc., La Jolla, CA, USA) was used to process the ELISA data, such as EC_{50} calculations. MicroCal Origin (version 7.0, Origin-Lab Corporation, Northampton, MA) was used to calculate the T_m values. The statistical analysis was performed by SPSS for Windows (version 13.0, IBM Corporation, Armonk, New York, USA) and one-way ANOVA was applied to all data unless otherwise stated.

3. Results

3.1. Effect of thimerosal on the antigenicity of HPV VLPs

As part of the antigen characterization with respect to alteration owing to thimerosal treatment (Fig. 1C), and the change in binding activity between the mAbs (in the mAb panel) and the antigens upon thimerosal treatment was analysed by a direct-binding ELISA. Fig. 2A illustrates the representative binding profiles of anti-HPV16 mAbs, which were grouped into three categories based on the fold changes in binding activity due to thimerosal treatment. For each type of the four HPV VLPs, a substantial portion of mAbs (shown in black) lost all binding activity due to thimerosal treatment (Fig. 2B). Using more than a two-fold reduction ($rEC_{50} > 2$) in antigen binding as a criterion, the conformational sensitivity of mAbs represents 23% (HPV6), 23% (HPV11), 38% (HPV16) and 47% (HPV18) in the whole mAb panels (Fig. 2C).

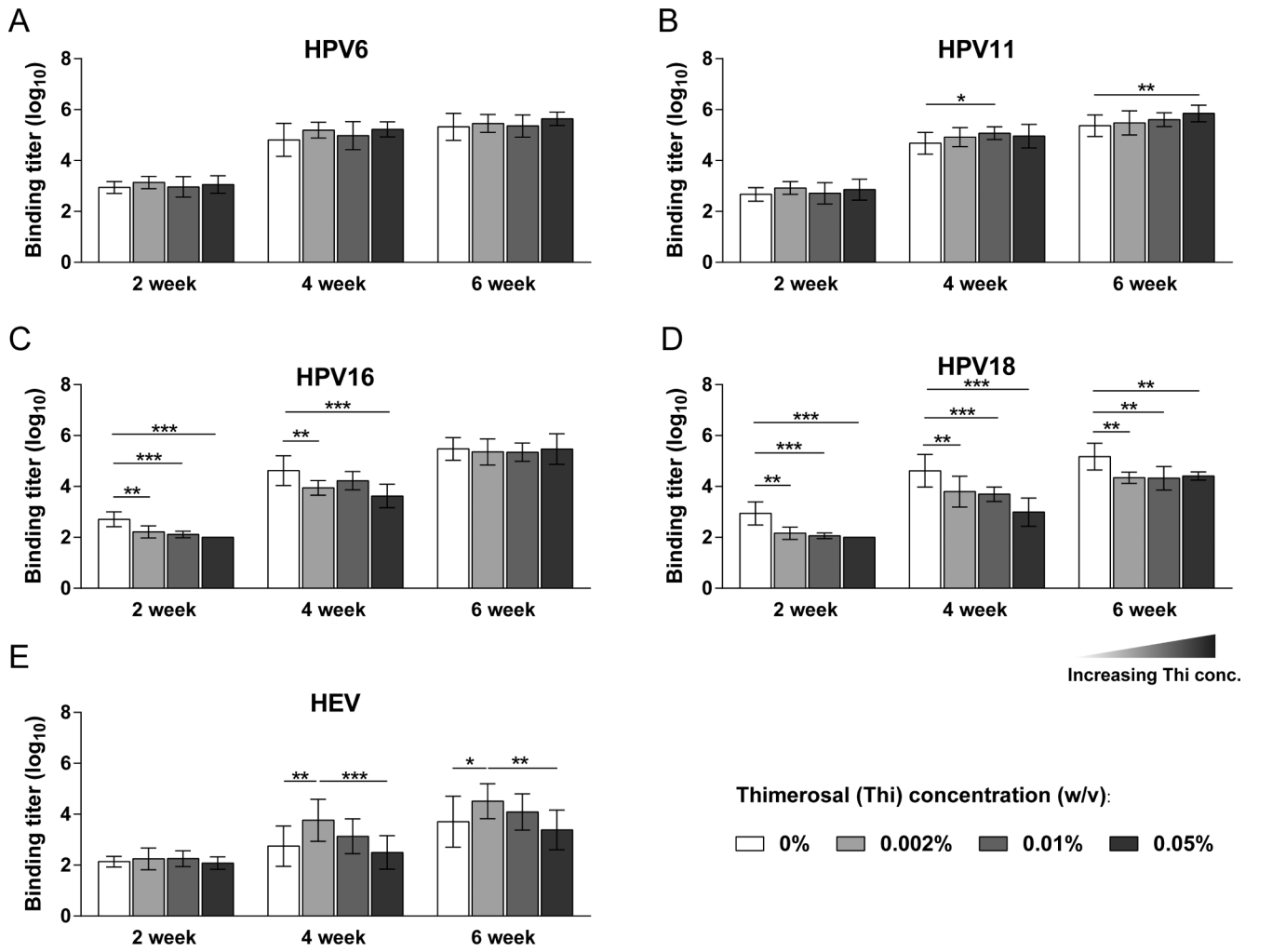

Fig. 4. Serum IgG binding titres for specific antigens after immunizing the mice with a pentavalent vaccine formulation. The sera harvested at 2, 4 and 6 weeks were analysed using an ELISA for binding titres against HPV6 (A), HPV11 (B), HPV16 (C), HPV18 VLPs (D) and HEV P239 (E). The end-point binding titre was the maximum dilution ratio that yielded a positive response compared with that of the cut off. The blank is the serum from mice that have not been immunized. The cut off was calculated by the average OD_{450} value of the blank plus three times s. d. (n = 10 for each group). The data are presented as the mean ± s.d.. The statistical analysis was performed by one-way ANOVA with the significance indicated as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$.

3.2. Effects of thimerosal and 2-PE on the conformational stability

The alteration in antigenicity of the test antigen is likely the result of conformational changes due to thiol modifications by thimerosal. The impact of thimerosal treatment on the conformational stability of five antigens was tested. The DSC profiles and T_m values for the five different antigens are shown in Fig. 3 and Table 1. Notably, the HPV18 antigen showed the most significant T_m value drop at approximately 30 °C, this indicates a marked destabilization of the protein conformation in the presence of thimerosal. The other three HPV antigens showed a less impressive reduction and had varying degrees of sensitivity, with T_m value changes of ~13.7–15.6 °C. As a control of 'inert' preservative, 2-PE was tested in parallel. As expected, a minimal change (ΔT_m value < 4 °C) in conformational sensitivity was observed in the presence of 2-PE for all the five test antigens. Considering that the adjuvant-adsorbed antigens were used in most vaccine formulations, the T_m values of aluminium-adsorbed antigens were also determined under the same experimental conditions (Supplementary Materials Fig. S1 and Table S1). The extent of the decrease in the T_m values for different antigens is generally consistent, regardless of whether the antigens are in an aqueous solution or are adsorbed onto particulate adjuvants. A good linear correlation was observed between ΔT_m (in solution) and ΔT_m (adjuvant-adsorbed) with a coefficient $R^2 = 0.937$ (Fig. 3H). Such a good agreement indicates that the VLP antigens maintained their conformation after adjuvant adsorption in the vaccine formulations [32,33].

3.3. Effect of thimerosal on immunogenicity

3.3.1. Binding titres

The mouse sera obtained after mouse immunization were used to quantify the end point titre of total IgG antibodies. As shown in Fig. 4, for the most affected HPV18 VLP, the binding titre of the high dose thimerosal group (0.05%) decreased by an average of ~25-fold compared with that of the control. Significant differences were always observed between the thimerosal-treated HPV18 groups and the control group at different bleeding times. Similarly, a downward trend was observed in the HPV16 group, and the binding titres decreased by ~6-fold in the thimerosal-containing groups after 4 weeks. By contrast, the immunogenicity of the HPV6 and HPV11 VLPs did not show much change in titres with or without thimerosal in the formulation.

3.3.2. Neutralization titres

PBNA for HPV serology is more relevant to antibody functions. Noticeably, the ability of the sera to neutralize pseudovirus was weakened significantly in the presence of thimerosal for HPV18 VLP. The

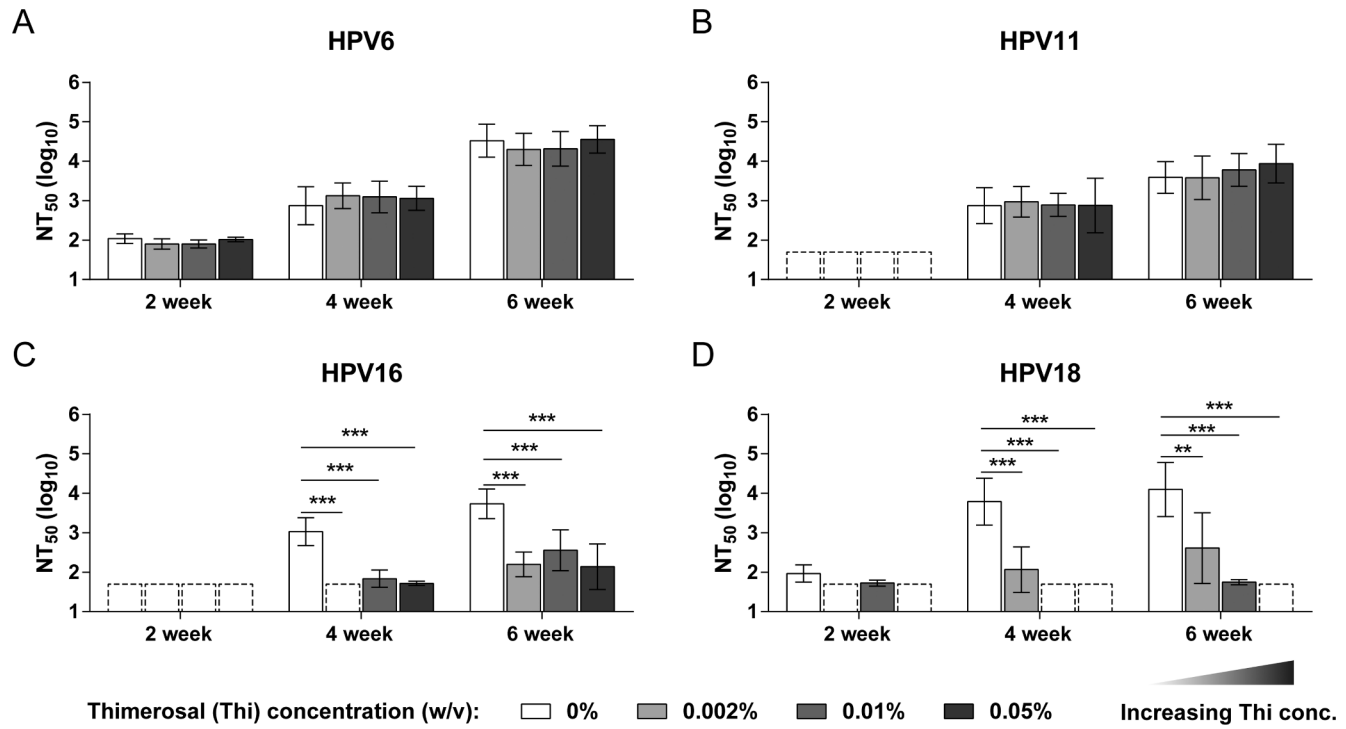

Fig. 5. Neutralizing antibody titres of mouse sera against specific pseudovirus particles were used to quantitate the functional antibody level that were elicited by different formulations. After immunization with pentavalent vaccine formulations, the mice were bled at 2, 4 and 6 weeks. The sera were used to perform PBNA to quantify the NT_{50} values against pseudovirus 6 (A), pseudovirus 11 (B), pseudovirus 16 (C) and pseudovirus 18 (D). The half neutralization titre (NT_{50}) was defined as the highest dilution of serum with a 50% inhibition of pseudovirus infection. The bars shown in the dotted line indicate that the neutralization activity of this given group was not detectable at the lowest dilution. Each sample was repeated in duplicate in one cell plate, and the data are presented as the mean ± s.d. (n = 10 for each group). The statistical analysis was performed by one-way ANOVA with the significance indicated as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$.

Table 2
Characteristics of functional mAbs used in the epitope specific assay. Horseradish peroxidase labelled mAbs were used in the assay with and without the competing antibodies in the sera after immunization with various formulations.

mAb	Source	Subclass	NC_{50}[a], ng/mL	Note	Ref.
HEV-8C11	Murine	IgG1	–	Neutralizing HEV in the rhesus monkey	[67,68]
HPV6-5D3	Murine	IgG2b	3.5	Recognizing immunodominant epitopes	[69]
HPV11-14A6	Murine	IgG1	1.9	Recognizing immunodominant epitopes	[21]
HPV16-26D1	Human	IgG1	0.6	V5-like mAb from human origin	[26]
HPV18-2G4	Murine	IgG2a	0.8	Recognizing immunodominant epitopes	–

[a] Half neutralization concentration (NC_{50}): effective mAb concentration that can prevent 50% of the pseudoviruses from infecting the cells.

serum neutralizing titre was below the test threshold in the middle (0.01%) and high (0.05%) concentrations of the thimerosal groups, and the NT_{50} of the low (0.002%) thimerosal group decreased by 35-fold in 4 weeks (Fig. 5). Similarly, the functional antibody activity of the HPV16 groups decreased significantly in the presence of a high concentration of thimerosal (0.05%), and the neutralization titre showed an evident decrease by ~24-fold in the same bleeding time with HPV18. In contrast, there were no significant differences in the functional antibody levels of the HPV6 and HPV11 groups in the presence of thimerosal.

3.4. Effect of thimerosal on an epitope-specific immunoassay

The epitope specificity of the serological response of immunized mice was assessed by inhibition ELISA with mAbs as indicators for quantifying a subset of antibodies that were elicited by immunization. These functional mAbs used in the assay were shown to recognize immunodominant epitopes on antigens (Table 2). As shown in Fig. 6, a sharp drop was observed in a dose-dependent manner in the inhibition rate of the sera in the HPV18 group when the mice were immunized with thimerosal-containing formulations. The inhibition rate of the sera from the low dose (0.002%) group decreased from 75.5% (control) to 41.0% due to the presence of thimerosal. More strikingly, the ability of sera to inhibit the mAb response was abrogated in the medium-dose (0.01%) and high-dose (0.05%) dose groups. For HPV16, the inhibition rates of sera decreased from 73.8% (control) to less than 50.0% in all thimerosal-containing groups. Similar to the results of the binding titre and neutralization titre, no significant impact was observed on the inhibition rate for the other three antigens (HEV, HPV6, and HPV11) in the presence of thimerosal.

3.5. Comparative structural analysis on HPV18 VLPs with or without thimerosal treatment

A structural analysis was performed to ascertain the impacts of thimerosal on the capsid structure since the changes in antigenicity and immunogenicity were quite pronounced. HPV18 VLP, which had its immunogenicity greatly affected by thimerosal, was chosen for this comparative analysis by cryo-EM. The cryo-EM micrographs of the HPV18 capsid proteins showed no obvious difference between the non-treated and thimerosal-treated samples, and both samples maintained their spherical particle morphology (Fig. 7A). A 3D reconstruction of

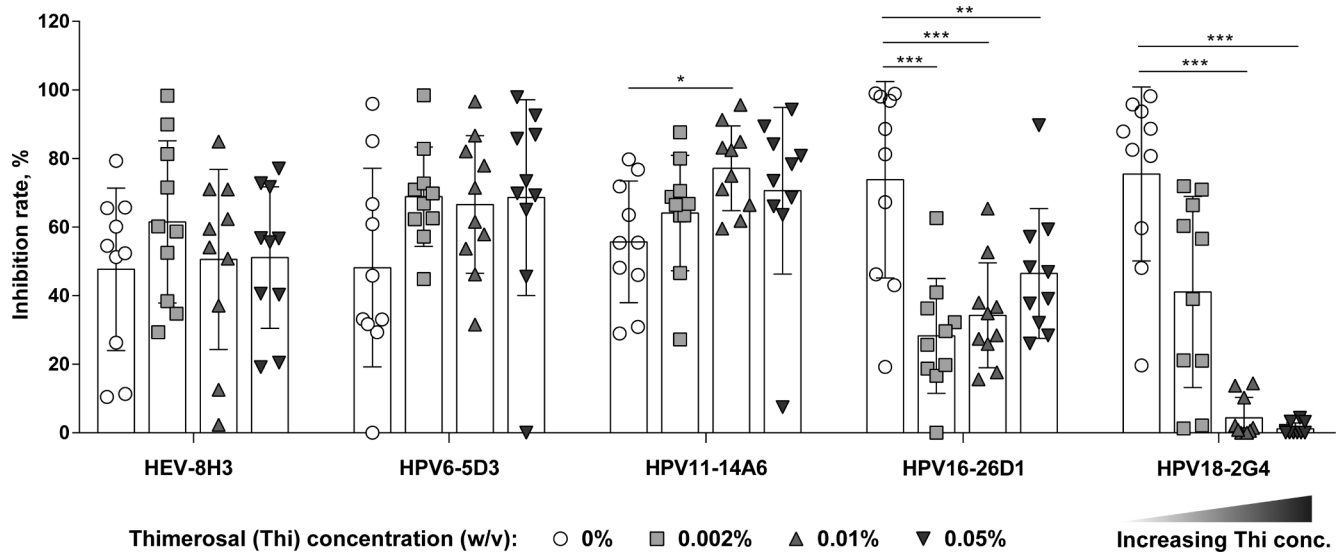

Fig. 6. Epitope-specific assay for an immunogenicity assessment using an inhibition ELISA. The sera collected at 10 weeks (diluted 1:100) were used to compete with five specific mAbs in an inhibition ELISA. The mAbs used here have good neutralization abilities and are able to identify immunodominant epitopes on antigens. The inhibition rate was calculated as [OD_{450} (non-inhibited well) − OD_{450} (inhibited well)]/OD_{450} (non-inhibited well) × 100%. Each sample was repeated in duplicate in one plate, and the data are presented as the mean ± s.d. (n = 10 for each group). The statistical analysis was performed by one-way ANOVA with the significance indicated as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$.

selected particles yielded the structures of the control and thimerosal-treated HPV18 VLPs at 11.5 Å and 13.0 Å, respectively, with a comparable number of micrographs included for the parallel 3D reconstruction processes (Supplementary Materials Table S2). The two density maps both showed a T = 7 icosahedral capsid structure with similar diameters between 58 and 60 nm (Fig. 7B), with the thimerosal-treated VLP being slightly larger. Notably, the superposition of the two maps revealed some structural differences between the two samples (Fig. 7C). Compared with the structure of the non-treated HPV18 VLP, which is similar to some other reported HPV capsid structures [32], the thimerosal-treated HPV18 VLP structure was slightly less compact, showing expanded dimensions of approximately 2%. Most strikingly, an obviously conformational rearrangement of each pentamer, particularly near the central hole in each of the pentamers, was observed due to the thimerosal treatment. This rearrangement resulted in the closing of the central channel in the inner side of each pentamer. Such significant structural alterations could be due to the thiol modifications in the thimerosal-treated HPV18 VLP.

4. Discussion

While thimerosal has been a widely used vaccine preservative for decades, currently, most vaccines nowadays are supplied in single-dose vials without preservatives. However, the manufacturing of single-dose vials inevitably increases the costs and the demand on cold chain capacity, which is scarce in low-income countries [34,35]. Under these circumstances, multi-dose vials containing preservative are more cost-effective [36,37]. Additionally, thimerosal-containing vaccines are still used in developed countries. Approximately half of the licensed influenza vaccines used in the United States still contain thimerosal [38,39]. Accordingly, the use of thimerosal was recently studied as a preservative in some candidate vaccines, such as an investigational rotavirus vaccine and a meningococcal vaccine [40–42]. Thus, understanding the impacts of thimerosal on the physico-chemical characteristics and functional attributes of antigens is still needed, even after its long clinical use.

Thimerosal is not fully compatible with all antigens, particularly for Cys-containing proteins. As early as 1994, a report described the deleterious effects of thimerosal on the potency/antigenicity of a poliovirus vaccine [43]. More recently, the presence of thimerosal was shown to cause a partial loss of D-antigens on poliovirus types 1–3 in the antigenicity assessment of the vaccine [44]. In an investigational vaccine, a decrease in the antigenicity and enthalpy of protein unfolding were observed in a recombinant protein based rotavirus vaccine, which considered thimerosal as the preservative [45]. These observations are in line with the alterations in antigenicity, immunogenicity and the protein conformational stability illustrated in this study.

The physico-chemical characterization of antigens is critical for vaccine process development, formulation and product stability. The most important feature of prophylactic vaccines is the capacity to elicit robust antibody titres *in vivo*. However, the high binding titre of antibodies for a given immunogen does not necessarily correspond to the desired functionality. In the initial clinical study of HPV vaccines, the thimerosal-containing vaccine hardly elicited neutralizing antibodies in human vaccinees, despite the high binding titres detected in the sera [46]. The surprising results were partly attributed to the presence of thimerosal in the formulation without thorough investigation [47]. Any binding of antibodies to surface-exposed epitopes on antigens can be detected in a simple binding-based ELISA, regardless of whether the epitopes are functional [48]. The *in vitro* PBNA can more faithfully mimic the interaction of antibodies with viruses and can reflect the antibody levels based on the viral neutralization activity [49,50]. By assessing the ability of sera to neutralize pseudoviruses, the level of functional antibodies elicited by vaccines can be measured quantitatively.

As the gold standard method for HPV serological assays, PBNA measures the effectiveness of the sera with all kinds of antibodies with neutralizing activity. Epitope-specific assays, e.g., by the inhibition ELISA as described here, have advantages in that they help us determine the exact nature or footprints of the functional antibodies. Only a subset of the elicited antibodies, having similar footprints with the tracer mAbs would register in the assay. Such competition/inhibition immunoassays were used in the clinical development stages of Gardasil, and they are still being used to support the development and the post-licensure studies of the second-generation vaccine Gardasil-9 [51–53]. For instance, V5 was used as a tracer mAb in the HPV16 serology to measure the antibodies in the sera that share the same V5 epitope on

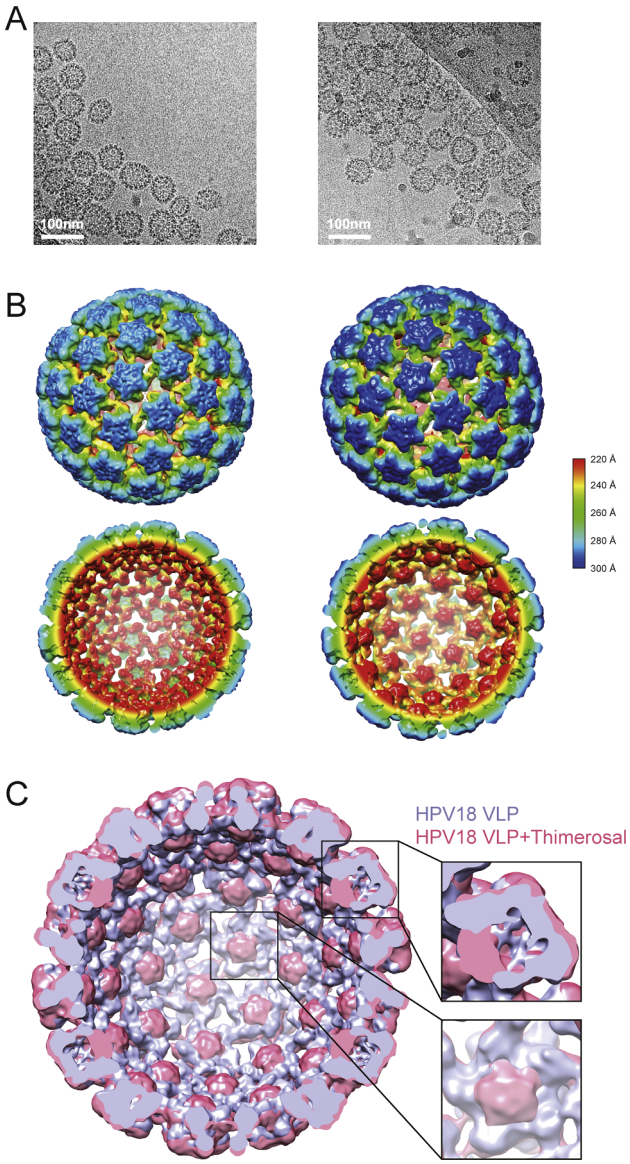

Fig. 7. Comparative analysis of the structural changes of HPV18 VLP upon thimerosal treatment. A cryo-EM 3D reconstruction for the control and thimerosal-treated HPV18 VLPs was performed with a comparable 831 micrographs. (A) The cryo-EM images of the non-treated (*left*) and the thimerosal-treated (*right*) HPV18 particle capsids are shown. (B) The iso-contoured views of the cryo-EM maps of the control (*upper left*) and the thimerosal-treated (*upper right*) HPV18 VLPs. The density maps were viewed along the icosahedral 2-fold axis and were coloured radially. The central sections of the corresponding maps are displayed in the lower row. (C) The superimposed density maps of non-treated (*violet*) and thimerosal-treated (*pink*) HPV18 VLPs. The black squares show the conformational differences of particles from two samples. The electron microscopy density maps for HPV18 VLP and thimerosal-treated HPV18 VLP have been deposited in the Electron Microscopy DataBank (EMDB) under EMD-9920 and EMD-9921, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the capsid of HPV16 [46]. In our study, 26D1, a V5-like human mAb, and 2G4, a neutralizing murine mAb, were used in an epitope-specific serology assessment of HPV16 and HPV18. The reduced ability of the elicited antibodies in the sera against both HPV 16 and HPV18 to inhibit the tracer mAbs was detected in the sera of immunized mice; this indicates that the epitopes on the VLP antigens that are recognized by these mAbs were impaired in the presence of thimerosal in vaccine formulations.

Since these different HPV VLP types all contained Cys residues (Fig. 1B), the observed alteration in antigenicity and immunogenicity was likely due to the structural changes owing to the thiol modification in the presence of thimerosal. Discernible structural changes of the HPV18 VLPs were observed by 3D reconstruction in the current study; this is in agreement with the striking differences observed in antigenicity and immunogenicity for HPV18. Similar to the impact on purified recombinant virus-like particles, the conformation of L1- and L2-containing HPV16 pseudovirions in cell culture was altered and accompanied by a decrease in infection capacity after incubating with thiol-reactive reagents [54].

The interaction of a free protein thiol and ethyl mercury (the hydrolysate of thimerosal) is the mode of action for its anti-bacterial activity in an aqueous solution [55]. In biological environments, thimerosal quickly reacts with thiols on critical cellular components, leading to a limited thiol availability [56–58]. On the molecular level, the structure of the polypeptide chain on bovine serum albumin was found to be affected by thimerosal treatment. The formation of an adduct between ethyl mercury and the free thiol on the Cys34 residue was identified and characterized [59]. The complete or partial thiol modification on certain Cys residues is a potential mechanism for the thimerosal effect on Cys-containing proteins. Noticeably, an apparent correlation between the negative effect of thimerosal on antigen immunogenicity and the number of Cys residues contained in HPV L1 was observed, with HPV18 being the greatest sensitive type to thimerosal treatment and the type with the most number of Cys residues (14 Cys residues in HPV 18, Fig. 1B). For HPV18 VLP, a specific chemical modification of thiol will be investigated in the future since dramatic impacts were observed on antigenicity and immunogenicity coupled with a significant alteration on the capsid structure near the inside central hole.

Although the presence of thimerosal may lead to structural and functional alterations in antigens, thimerosal is still the most widely used vaccine preservative in multi-dose vaccines and in some influenza vaccines. Specifically, thimerosal-containing vaccines are still used in more than 120 countries and have been used to vaccinate over 84 million children, saving approximately 1.4 million lives annually [60,61]. For vaccines with thiol-containing antigens, alternative preservatives, such as 2-PE, should be considered. Currently, 2-PE has been used in IPOL (an inactivated poliovirus vaccine) and it is more bacteriostatic and more economical than thimerosal in some vaccines [62–66]. Our results also show a minimal effect of 2-PE on the conformational stability of HPV antigens, suggesting that 2-PE is a candidate preservative for thiol-containing antigens when the addition of a preservative becomes necessary.

5. Conclusion

The impact of thimerosal treatment on a pentavalent vaccine was quantitatively assessed. The binding activity between the HPV antigens and the mAbs was altered to different degrees in the presence of thimerosal. Lower protein conformational stability was reflected by the transition temperature during thermal unfolding for the thimerosal-treated HPV antigens. The immunogenicity of HPV16 and HPV18 in mouse studies was observed to decrease more significantly (compared to HPV6/11), which is reflected by the lower binding and functional antibody levels as well as the lower epitope-specific antibody levels. With cryo-EM 3D reconstruction, structural alterations were observed in HPV18 VLPs as a result of thimerosal treatment. Thus, the choice of vaccine preservatives should be carefully considered, particularly if the antigen contains thiol, in vaccine research and development where the addition of a preservative is deemed necessary.

Declaration of Competing Interest

Huirong Pan and Zhijie Lin are employees of the Xiamen Innovax

Biotech Company.

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Appendix A. Supplementary material

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