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Original article

Capsid destabilization and epitope alterations of human papillomavirus 18 in the presence of thimerosal

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ABSTRACT

Thimerosal has been widely used as a preservative in drug and vaccine products for decades. Due to the strong propensity to modify thiols in proteins, conformational changes could occur due to covalent bond formation between ethylmercury (a degradant of thimerosal) and thiols. Such a conformational change could lead to partial or even complete loss of desirable protein function. This study aims to investigate the effects of thimerosal on the capsid stability and antigenicity of recombinant human papillomavirus (HPV) 18 virus-like particles (VLPs). Dramatic destabilization of the recombinant viral capsid upon thimerosal treatment was observed. Such a negative effect on the thermal stability of VLPs preserved with thimerosal was shown to be dependent on the thimerosal concentration. Two highly neutralizing antibodies, 13H12 and 3C3, were found to be the most sensitive to thimerosal treatment. The kinetics of antigenicity loss, when monitored with 13H12 or 3C3 as probes, yielded two distinctly different sets of kinetic parameters, while the data from both monoclonal antibodies (mAbs) followed a biphasic exponential decay model. The potential effect of thimerosal on protein function, particularly for thiol-containing proteinaceous active components, needs to be comprehensively characterized during formulation development when a preservative is necessary.

1. Introduction

Thimerosal, due to its bacteriostatic and fungistatic properties, has been widely used as a preservative in formulations for biologics and drug products [1–3]. In the late 1990s, concerns were raised about whether thiomersal-containing vaccines might contribute to the development of autism and other neurodevelopmental disorders in children. However, no definitive evidence from reputable scientific studies was available to support these claims [4–7]. The WHO Consultation (April 3–4, 2012) reaffirmed the view that the benefits of using thiomersal-containing multi-dose vaccines far outweighed any risks [8]. Currently, thimerosal is still in use in several licensed vaccines, including meningococcal and multiple-dose seasonal influenza vaccines (Table S1), and other biological products [3].

Having been widely used for decades as a vaccine preservative, thimerosal had been thought to be inert with respect to vaccine potency until the last 10–20 years [9]. An earlier study by Sawyer

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et al. [10] showed that thimerosal had deleterious effects on the potency of inactivated poliovirus vaccine (IPV) as measured by monoclonal antibody (mAb)-based enzyme-linked immunosorbent assay (ELISA), but the overall potency did not change when using a polyclonal antibody preparation. The ELISA potency based on mAbs of all three poliovirus antigens in the presence of thimerosal showed a decrease to different degrees for the different types over the observation period [10]. These studies provided the first report on the epitope-specific loss of antigenicity of a vaccine product in the presence of thimerosal. In addition, Kraan et al. [11] found that thimerosal negatively affected the antigenicity of lyophilized inactivated poliovirus vaccine by ELISA using a mixture of serotype-specific anti-poliovirus mAbs. These results showed that vaccine preservatives might result in the partial or even complete loss of antigenicity of vaccine antigens when monitored with specific mAbs. While the negative impacts on vaccine antigenicity and immunogenicity were observed, the time course of such an alteration has been poorly understood in the vaccine formulations.

The presence of the correct conformation of protein antigens is the structural basis for vaccines to elicit functional antibodies and to confer immunity against infectious diseases. Thus, thimerosal as a preservative in vaccine formulations should be compatible with the antigens for maintaining the virion-like epitopes. Human papillomaviruses (HPVs), especially HPV16 and HPV18, are the two most common high-risk HPV types that cause cervical cancer [12]. Currently, several HPV prophylactic vaccines based on virus-like particles (VLPs) have been licensed against HPV infection [13]. It has been reported that the ELISA titres in human sera of HPV VLP-based vaccine (types 16/18) preparations showed an increase upon immunization. However, the thimerosal-containing vaccine failed to induce neutralizing antibody responses [14]. In our previous study, using a mouse model, the immunogenicity of the HPV16 and HPV18 VLPs antigens in the formulation was shown to be significantly decreased in the presence of thimerosal [15].

In this study, a panel of murine mAbs against HPV18 VLPs was assessed for its sensitivity with respect to thimerosal treatment. Two representative mAbs with the highest sensitivity to thimerosal treatment, 13H12 and 3C3, were chosen to perform the in-depth characterization of the epitope alteration with respect to the time scale of antigenicity loss and thimerosal concentration dependence. The kinetics analyses of HPV18 VLP antigenicity loss yielded unique information on the kinetic properties for the changes in immunoreactivity to these two mAbs. In addition, the decrease in binding activity to different mAbs of HPV18 VLP antigens adsorbed on aluminium-based particulate adjuvants in the presence of thimerosal was visualized using fluorescence imaging-based high content analysis.

2. Materials and methods

2.1. Recombinant HPV18 VLPs

The recombinant HPV18 L1 protein was expressed in *Escherichia coli* from Xiamen Innovax Biotech (Xiamen, China). The recombinant HPV18 VLPs were produced and purified according to the previously published procedures [16]. The concentration of HPV18 L1 protein was measured using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin as a standard.

2.2. Monoclonal antibodies

A total of 26 in-house anti-HPV18 VLPs mAbs were produced from hybridoma cell lines, provided by Xiamen Innovax Biotech and purified with a Protein A column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The concentration of the purified mAbs was determined by Ultrospec 2100 pro UV/Visible Spectrophotometer (GE Healthcare, Piscataway, NJ, USA) at 280 nm (OD 1.4 for a 1 mg/mL IgG solution). The purified 3C3 mAb was labelled with horseradish peroxidase (HRP) by a periodate conjugation method as previously reported [17].

2.3. Human and mouse serum samples

The serum samples derived from humans and mice for this study were reported previously [15,18]. Briefly, human serum samples (n=8) were collected from volunteers immunized with three doses of an investigational bivalent (HPV16/18) vaccine (Xiamen Innovax, Xiamen, China). Human serum samples 1–8 were numbered as 12, 1, 27, 5, 4, 29, 18, and 26 described by Zhang et al. [18]. Mouse serum samples (n=8) were collected from mice after 6 weeks of immunization with three doses of a thimerosal-free formulation of a pentavalent vaccine (HPV6/11/16/18/HEV). All sera-based studies were carried out in accordance with the guidelines of The Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Xiamen University Laboratory Animal Management Ethics Committee (XMU-LAC20160032) and Ethics Committee of Jiangsu Provincial Center for Disease Prevention and Control (JSJK2019-A006-02).

2.4. Morphology and particle size of the VLPs

Thimerosal (AR, ≥98.0%, Lot No. F20110111) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). A concentration of 0.01% (*m/V*) was the most commonly used concentration for thimerosal in vaccine formulations (Table S1). HPV18 VLPs (0.1 mg/mL) were treated with 0.01% (*m/V*) thimerosal for 24 h at 4 °C and then treated with the same concentration of native HPV18 VLPs, applied to a carbon-coated grid and stained with 2% (*m/V*) uranyl acetate after removal of excess fluid. The morphologies of the HPV18 VLPs were examined using a JEM2100HC transmission electron microscope (JEOL, Tokyo, Japan) operated at 120 kV.

2.5. Thermal stability by differential scanning fluorimetry (DSF) and cloud point analysis

DSF [19,20] was carried out by a real-time PCR instrument, Bio-Rad T100™ Thermal Cycler (Hercules, California, USA). First, 200 μg/mL HPV18 VLPs were treated with different concentrations (0%, 0.001%, 0.005%, 0.01%, 0.02%, and 0.05% (*m/V*)) of thimerosal for 24 h at 4 °C. The fluorescent dye, SYPRO Orange (Sigma–Aldrich, St. Louis, MO, USA), was diluted 50× from its stock concentration using deionized water and then mixed at a volume ratio of 1:9 of the antigens to thimerosal. Subsequently, 50 μL of the mixed solution was added to each well of the plate. Then, the PCR plate was sealed and centrifuged at 1000 rpm for 1 min. The real-time PCR machine was used to heat the samples at intervals of approximately 0.5 °C ramping from 10 °C to 90 °C. Fluorescence emission was collected using a HEX filter (560 nm–580 nm). Melting curves of fluorescence intensity at 570 nm were obtained with a Bio-Rad CFX Manager. The transition midpoint temperature (T_m) values were determined by derivative analysis using the software package OriginPro 9.1 (Origin Lab Corp., Northampton, MA, USA). Each sample was determined by triplicate measurements. The cloud point with temperature ramping was determined by turbidity measurements with a UV2100PRO, which was equipped with an external Peltier thermal controller in the temperature range of 30–90 °C. The samples were measured at a concentration of 0.1 mg/mL L1 protein. The optical density at 350 nm was used to detect the signals due to protein aggregation.

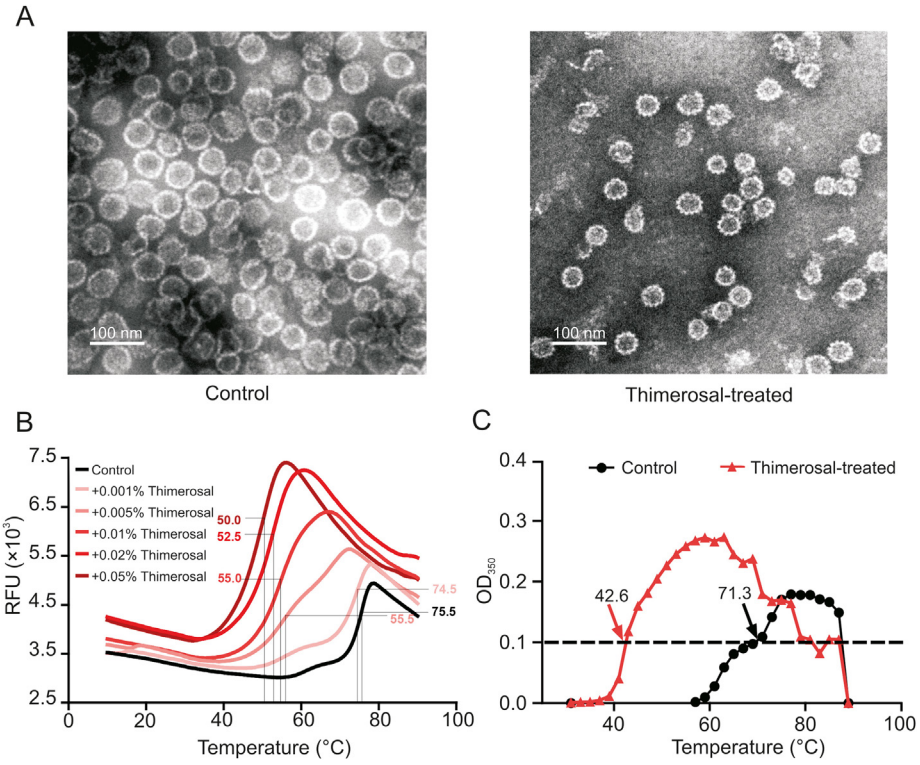

Fig. 1. Characterization of the morphology and thermal stability of HPV18 VLPs with or without thimerosal treatment. (A) The morphologies of the control and 0.01% (*m/V*) thimerosal-treated HPV18 VLPs. (B) The thermal stability of VLP antigens treated with different concentrations of thimerosal as monitored by DSF [19,20]. T_m differences were across the range from 50.0 °C (0.05% (*m/V*) thimerosal-treated) to 75.5 °C (control). (C) The cloud point temperatures of thimerosal-treated and control VLPs were approximately 43 °C and 71 °C, respectively.

2.6. Comparative study of polyclonal antibody binding activity to VLPs

First, 80 μg/mL HPV18 VLPs were pre-incubated in the presence of 0.01% (*m/V*) thimerosal for 24 h at 25 °C. Then, the microplates were coated at 25 °C for 2 h with 100 ng/well VLPs with or without thimerosal treatment. After plate blocking, the plates were incubated at 25 °C for 1 h with 100 μL of 2-fold serial dilutions of human serum and 150 μL of 3-fold serial dilutions of mouse serum diluted with assay diluent. After 5 wash cycles, a goat-anti-mouse or goat-anti-human IgG horseradish peroxidase (HRP) conjugated at 1:5000 (*V/V*) was added to the plates and incubated for 1 h at 25 °C. Subsequently, 100 μL per well tetramethyl benzidine substrate solution (Beijing Wantai Biological Pharmaceutical Co., Ltd., Beijing, China) was added and incubated for 10 min at 25 °C. The reaction was stopped by adding 50 μL of 2 M sulfuric acid per well, and the intensity in the well was measured at 450 nm against 630 nm as the background. The binding activity of the control or thimerosal treated VLPs was defined as the antibody dilution required to achieve 50% of the maximal signal (ED_{50}). The relative ED_{50} ($ED_{50\ (control)}/ED_{50\ (thimerosal\text{-}treated)}$) was used to measure the relative binding activity of the polyclonal antibody to HPV18 VLPs.

2.7. The mAb binding activity to the VLPs with or without thimerosal treatment

Direct binding ELISA was used to evaluate the median effective concentration (EC_{50}) of mAbs in solution against the surface-immobilized recombinant HPV18 VLPs with or without thimerosal treatment. Some VLPs (80 μg/mL) were treated with 0.01% (*m/V*) thimerosal for 24 h at 4 °C. Then, the microplates were coated at 25 °C for 2 h with 100 ng/well VLPs with or without thimerosal treatment. After plate blocking, the plates were incubated and maintained at 25 °C for 1 h with 100 μL of 2-fold serial dilutions (starting with 1 μg/mL mAbs) of 26 different mAbs using assay diluent. The binding activity of the control VLPs or thimerosal-treated VLPs was defined as the antibody concentration required to achieve 50% of the maximal signal (EC_{50}, ng/mL). The relative EC_{50} ($EC_{50\ (thimerosal\text{-}treated)}/EC_{50\ (control)}$) was used to measure the relative binding activity for mAbs to HPV18 VLPs coated on ELISA plates.

2.8. Surface plasmon resonance (SPR) for antigenicity analysis

The SPR technique was employed to assess the antigenicity of HPV18 VLPs using a Biacore 3000 instrument (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with similar procedures as previously described [21]. Briefly, 80 μg/mL VLPs were pre-incubated in the presence of thimerosal at different concentrations (0%, 0.001%, 0.005%, 0.01%, and 0.02% (*m/V*)), diluted to 20 μg/mL and detected in-cycle for 24 h at 25 °C. Two mAbs (3C3 and 13H12, 30 μg/mL) were captured by chemically immobilized GAM-Fc on the chip surface with HBS-EP running buffer (0.01 M HEPES, 0.5 M NaCl, 3 mM EDTA, 0.005% (*V/V*) Tween-20, pH 7.4). The HPV18 VLPs treated with thimerosal subsequently flowed through the chip surface binding to the captured mAbs. The relative antigenicity was calculated by normalizing the rRU (RU_{Ag}/RU_{Ab}) of the thimerosal-treated VLPs in a flow cell (0%, 0.001%, 0.005%, 0.01%, and 0.02% (*m/V*)) to that of the control HPV18 VLPs in a separate flow cell in the same assay cycle.

Table 1
Conformational stability analysis of HPV18 VLPs upon thimerosal treatment detected by DSF. The T_m values were measured in three independent replicates.

Treatment	T_m (°C)			Average	RSD (%)	$\triangle T_m$ (°C)[a]
	Rep-1[b]	Rep-2	Rep-3			
Control	75.5	75.5	75.5	75.5	0.00	
+0.001% thimerosal	74.5	74.5	74.5	74.5	0.00	−1.0
+0.005% thimerosal	55.5	56.0	56.0	55.8	0.42	−19.7
+0.01% thimerosal	55.0	55.0	54.5	54.8	0.43	−20.7
+0.02% thimerosal	52.5	52.5	52.5	52.5	0.00	−23.0
+0.05% thimerosal	50.0	50.0	50.5	50.2	0.47	−25.3

[a] $\triangle T_m$: the difference of T_m values between thimerosal treated and control samples.
[b] T_m values in "Rep-1" were derived from the traces shown in Fig. 1B.

2.9. Sandwich ELISA for VLP antigenicity analysis

Sandwich ELISA was utilized to determine the antigenicity of the HPV18 VLPs with 0.01% (*m/V*) thimerosal treatment compared to the control for 24 h at 4 °C. The 96-well micro-plate was coated with the mAb 13H12 as the capture antibody (100 ng per well) at 4 °C overnight. After plate coating and blocking, a set of eleven serial two-fold dilutions of HPV18 VLPs with or without thimerosal treatment, starting at 4 μg/mL, were added and incubated at 25 °C for 1 h. Then, 100 μL of mAb 3C3 labelled with HRP used as the detection antibody diluted 1:3000 (*V/V*) in assay diluent was added and incubated at 25 °C for 1 h.

2.10. The antigenicity of VLP thimerosal containing vaccine detected by imaging-based high content analysis (HCA)

An Opera Phenix high content system (PerkinElmer, Wellesley, MA, USA) and fluorescence plate reader (Beckman Coulter, Inc., Brea, CA, USA) were used for the antigenicity analysis of HPV18 antigens adsorbed on aluminium-based adjuvants. VLPs (160 μg/mL) were preincubated with 0.02% (*m/V*) thimerosal at 25 °C for approximately 30 min, and then the adjuvant was added to the antigen solution at a volume ratio of 1:1 and mixed sufficiently.

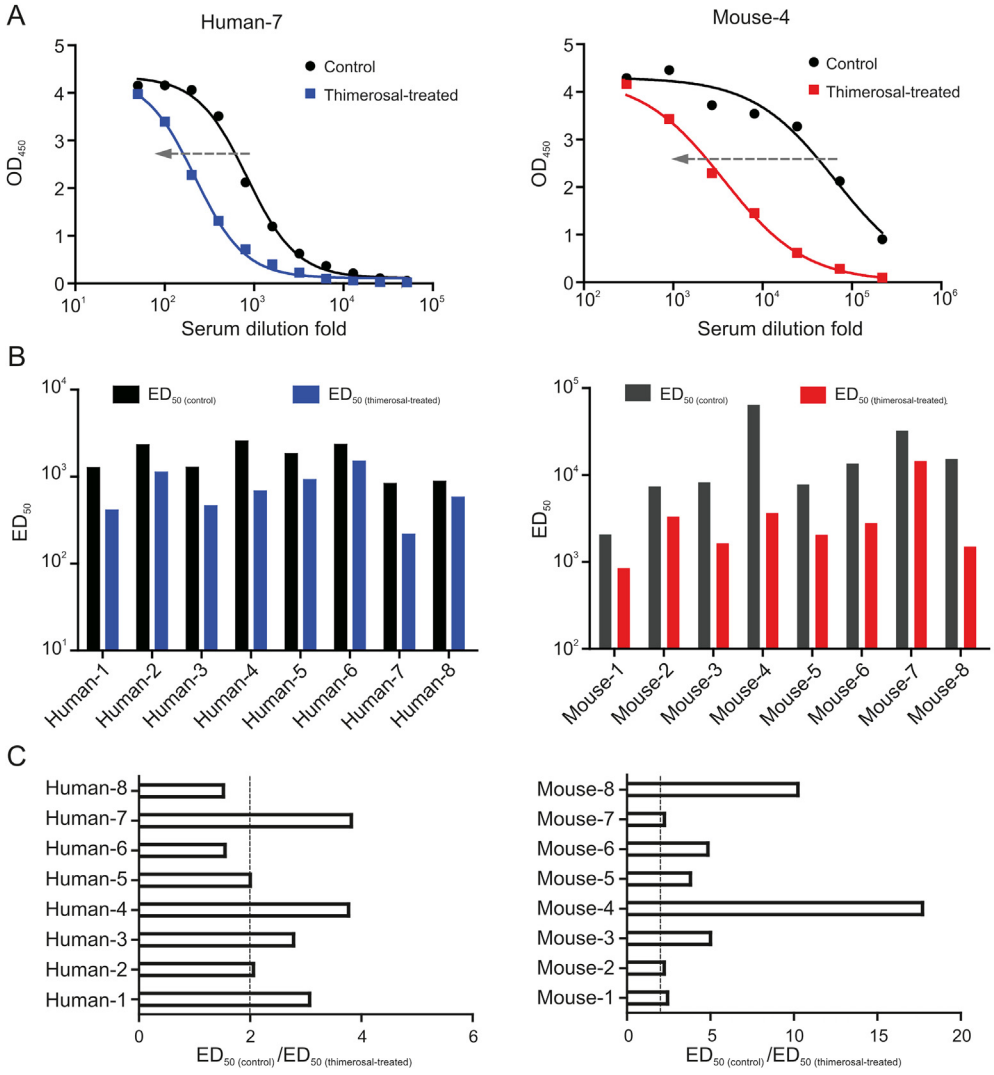

Fig. 2. The altered antigenicity of HPV18 VLPs to antibodies in human or mouse serum. Human and mouse serum samples were taken after post immunization using intact HPV18 VLPs based vaccines. (A) Human-7 and mouse-4 were the serum samples that showed the highest sensitivity to thimerosal treatment. The binding activities of eight different human and mouse serum samples to VLP antigens with and without thimerosal treatment are shown in Figs. S2 and S3. (B) Binding activity of eight different human and mouse serum samples to the control or thimerosal-treated VLPs (ED_{50}) which is the serum dilution fold required to achieve 50% of the maximal signal. (C) The relative binding activity ($rED_{50} = ED_{50\ (control)}/ED_{50\ (thimerosal\text{-}treated)}$) of eight different human and mouse serum samples to thimerosal-treated VLPs.

Table 2
Characteristics of a panel of HPV18 murine monoclonal antibodies and their relative binding activity to thimerosal treated VLPs ($rEC_{50} = EC_{50\ (thimerosal\text{-}treated)}/EC_{50\ (control)}$) compared to the control.

HPV 18 antibodies	Subclass	Epitope type[a]	Control EC_{50}[b] (ng/mL)	Thimerosal-treated EC_{50}[c] (ng/mL)	Sensitivity of thimerosal-treatment (rEC_{50})[d]
I. *Very sensitive to thimerosal treatment ($rEC_{50} > 4.0$)*					
13H12[e,f]	IgG_{2b}	C	35	>1000	28
3C3[f]	IgG_{2b}	C	28	642	23
5H2	IgG_1	C	45	900	20
11A9[f]	IgG_{2a}	C	23	394	17
II. *Sensitive to thimerosal treatment ($1.5 \leq rEC_{50} \leq 2.0$)*					
2H6	IgG_{2a}	L	500	>1000	2.0
14G5[f]	IgG_1	C	505	>1000	2.0
4D3-1	IgG_1	L	528	>1000	1.9
15E3	IgG_1	C	17	28	1.6
III. *Insensitive to thimerosal treatment ($0.5 \leq rEC_{50} < 1.5$)*					
10D11	IgG_{2a}	C	437	618	1.4
4F11	IgG_1	C	638	697	1.1
14E9	IgG_1	L	52	49	0.95
2H5-3	IgG_{2a}	L	7.6	6.3	0.83
3G3	IgG_{2a}	L	6.0	4.5	0.79
11F10-2	IgG_1	C	142	99	0.70
15D2	IgG_1	L	151	96	0.64
5H7	IgG_1	C	423	259	0.61
2A7	IgG_{2a}	L	6.9	3.5	0.50
IV. *Preferring thimerosal-treated HPV18 VLPs ($0 < rEC_{50} < 0.5$)*					
9F5	IgG_{2a}	L	1.6	0.8	0.49
11A3	IgG_{2a}	L	20	9.5	0.49
16E3	IgG_{2a}	L	36	17	0.47
10E8[f]	IgG_1	L	37	17	0.47
4H12-1	IgG_{2a}	L	14	6.0	0.44
5G1	IgG_1	C	530	205	0.39
2H5-2	IgG_{2a}	L	10	3.6	0.36
4F6-2	IgG_{2b}	C	730	261	0.36
1F8	IgG_1	C	115	39	0.34

[a] Epitope type via Western blotting; "C" means conformational and "L" means linear.
[b] EC_{50} values were derived from four-parameter logistics fits of the direct binding ELISA data. All derived EC_{50} values have a relative standard error (RSD) of ~15%–20% when determined in independent triplicates.
[c] "Thimerosal-treated EC_{50}" is shortened for the EC_{50} of thimerosal-treated HPV18 VLPs. An EC_{50} greater than 1000 was calculated as 1000. The HPV18 VLPs were treated with 0.01% (*m/V*) thimerosal (Hg, 25 μg/mL) at 4 °C for 24 h.
[d] The binding activity of mAbs to thimerosal-treated VLPs to mAbs was assessed in a direct binding ELISA. The relative binding activity was calculated by the EC_{50} of thimerosal-treated VLPs/EC_{50} of control particles ($rEC_{50} = EC_{50\ (thimerosal\text{-}treated)}/EC_{50\ (control)}$).
[e] Two mAbs (3C3 and 13H12), underlined, were the most sensitive to thimerosal treatment VLPs and they were used for more assays in this study.
[f] Refers to the neutralizing mAbs evaluated with the HPV pseudovirus system in 293FT cells [18,22].

Then, the solution was kept at 4 °C for 24 h before testing. The final concentration of thimerosal was 0.01% (*m/V*), and the concentration of Al was 420 μg/mL. Two murine mAbs, 13H12 and 3C3, were labelled by DyLight 488 Amine-Reactive Dye (Pierce/Thermo Scientific, Rockford, IL, USA) according to the instructions. These mAbs were used to detect the antigenicity of the VLP antigens in the fluorescence immunoassay. The antigenicity of antigens is based on the measurement of total fluorescence intensity of HCA or using a plate reader to measure the thimerosal-treated VLPs compared to the control.

2.11. Statistical analysis

EC_{50} values of ELISA and HCA data were calculated using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). For kinetics studies, a double exponential equation was fitted to the Biacore data using the software package OriginPro 9.1.

3. Results

3.1. The morphology and thermal stability of VLPs in the presence of thimerosal

The transmission electron microscope (TEM) images showed that the HPV18 VLPs with thimerosal treatment were morphologically similar to the control and did not show any observable VLP aggregates or significant deformation of the spherical particles (Fig. 1A). However, the thermal stability of the HPV18 VLPs upon thimerosal treatment was dramatically reduced as reflected by a lower transition temperature by DSF or a lower cloud point for thermally induced aggregate formation. T_m of the control group was 75.5 °C, while the T_m for thimerosal-treated HPV18 VLPs decreased from 74.5 °C to 50.2 °C (Table 1, Figs. 1B and S1), a reduction of 25.3 °C in the transition temperature (Table 1). The cloud point was defined as the temperature when the discrete VLPs began to agglomerate as monitored at a given wavelength by the light scattering signals due to larger particle formation. The cloud point temperatures of HPV18 VLPs with or without thimerosal treatment were approximately 43 °C and 71 °C, respectively (Fig. 1C). The observed difference of 28 °C indicated that the VLPs treated with thimerosal have a much stronger propensity to aggregate during heat stress, indicating dramatic destabilization of the recombinant viral capsid.

3.2. Polyclonal antibody binding activity to VLPs with thimerosal treatment

HPV18 VLPs antigens as coating antigens, with or without thimerosal treatment, were used to measure the binding activity of the serum samples from animals or humans immunized with control VLPs. The activity of the polyclonal antibodies in the serum samples was shown to decrease to varying degrees upon treatment

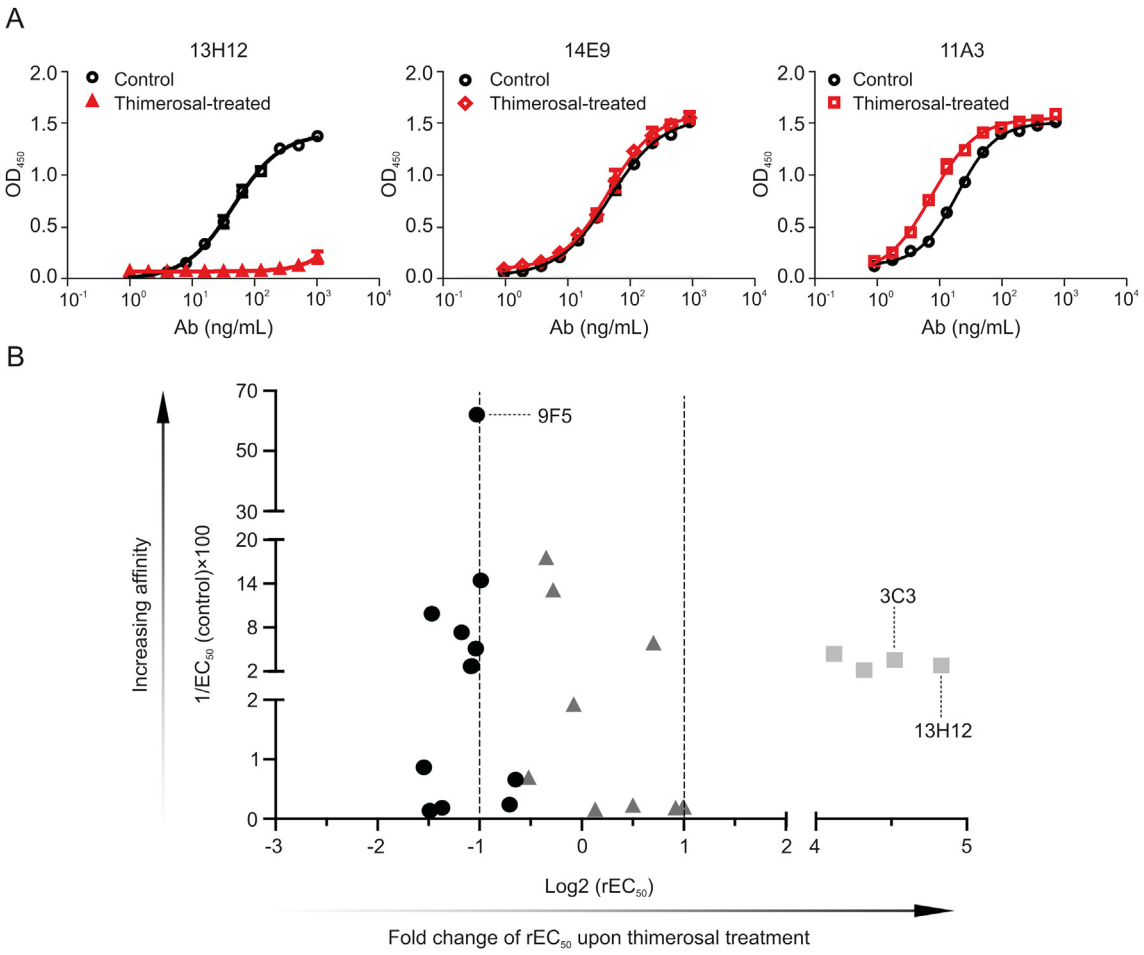

Fig. 3. The amplitude of changes to coating antigens reflected upon thimerosal treatment. The binding ability of HPV18 VLPs with or without thimerosal treatment to the mAbs was measured by direct binding ELISA. (A) The binding profiles of three representative mAbs (13H12, 14E9 and 11A3) to 0.01% (*m/V*) thimerosal-treated HPV18 VLPs compared to the control are shown. (B) The correlation between the binding affinity and the sensitivity to thimerosal treatment of VLPs for different mAbs. This figure was derived from Figs. S4A and B.

with the coating antigens (Figs. 2A and B, S2 and S3). The amplitude of change was more pronounced for the mouse serum samples. When compared to the control coating antigen, the relative binding activity (rED_{50}=$ED_{50\ (control)}$/$ED_{50\ (thimerosal\text{-}treated)}$) of the thimerosal-treated antigen on the plate showed an average 6-fold reduction in the mouse serum group and a 2.5-fold decrease in the human serum group (Fig. 2C). These results indicated that the epitopes in the recombinant HPV 18 capsid are somehow altered upon thimerosal treatment, lowering the binding of native antigen-elicited antibodies when the assays were performed in parallel using two different coating antigens.

3.3. The effects of thimerosal on VLP antigenicity

3.3.1. Varying degrees of mAb sensitivity to thimerosal-treated HPV18 VLPs

As shown in Table 2 [18,22] and Fig. S4A, the degree of sensitivity of a panel of 26 mAbs to thimerosal-treated HPV18 VLPs was ranked and categorized into four different groups according to the corresponding fold change in relative EC_{50} (rEC_{50} = $EC_{50\ (thimerosal\text{-}treated)}$/$EC_{50\ (control)}$) values. The majority of mAbs were shown to have decreased (Groups I and II in Table 2) or similar binding activity (Group III in Table 2) upon thimerosal treatment. In particular, upon thimerosal treatment, the binding activity of 13H12 and 3C3 to HPV18 VLPs showed a reduction of up to 20- to 30-fold. A small portion of the mAbs showed a slight increase in binding activity to thimerosal-treated VLP antigens (e.g., 11A3 in Fig. 3A and Table 2). Not surprisingly, the sensitivity to thimerosal-treated VLPs showed no apparent correlation to their affinity to VLP antigens (Fig. S4B), as the thimerosal-treated changes may be unique for any given mAb due to the nature and uniqueness of the epitope it recognizes.13H12 and 3C3, the two VLPs highly sensitive to thimerosal treatment, are also elite virus neutralizers (NC_{50} < 10 ng/mL), top binders to the VLPs and highly conformation-dependent. This would suggest that the epitopes sensitive to thimerosal treatment are also likely to be clinically relevant epitopes. In contrast, 9F5, a high affinity antibody with no virus neutralization capacity, exhibited no sensitivity to thimerosal-treated VLPs (Fig. 3B).

3.3.2. Thimerosal concentration dependence of the antigenicity reduction

As presented in Figs. 4A and B, the OD_{450} curves clearly showed a time-dependence (up to 48 h tested) and thimerosal concentration dependence. However, the decreasing trends in antigen binding to two mAbs (3C3 and 13H12) appeared to be unique for each mAb (Fig. 4B). For 3C3, the plateauing effect appeared at higher thimerosal concentrations. These results indicated that mAb

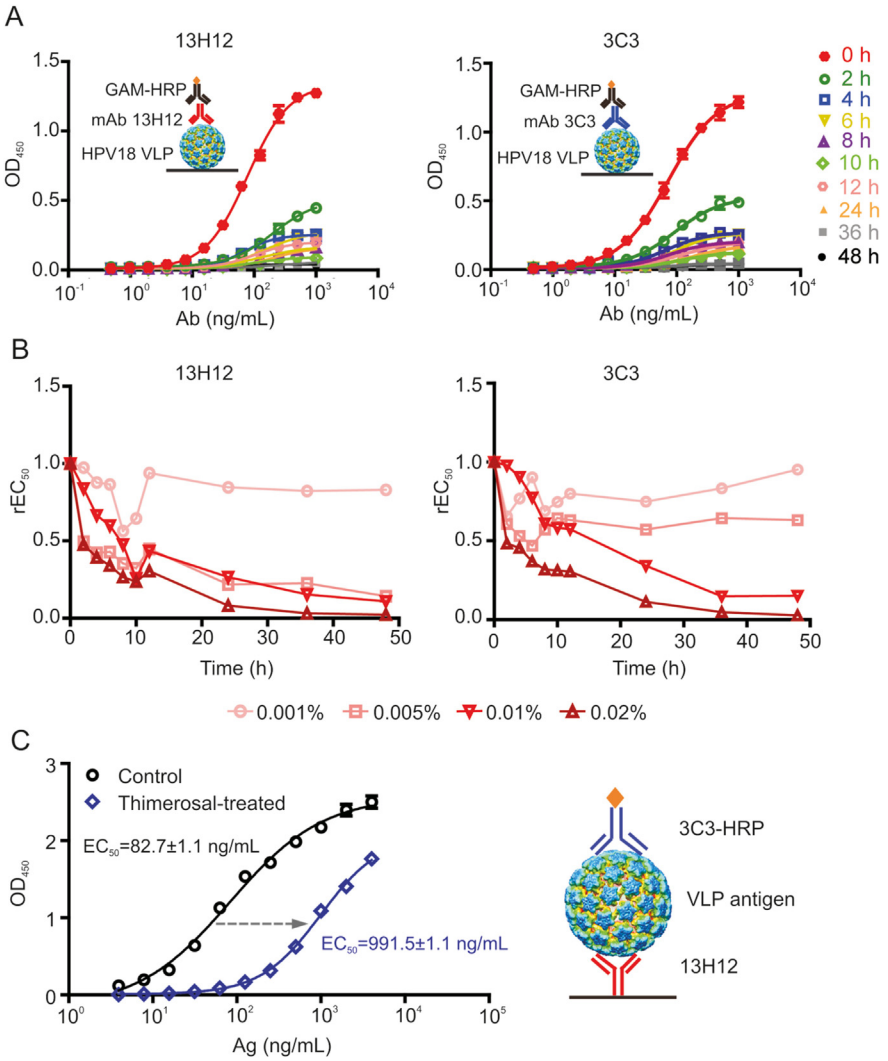

Fig. 4. The ELISA binding activity of two mAbs (13H12 and 3C3) to VLP antigens after pretreatment with thimerosal. (A) The binding profiles of two mAbs (13H12 and 3C3) to VLP antigens treated with 0.01% (*m/V*) thimerosal at different time points. (B) rEC_{50} values at different processing time points treated with different thimerosal concentrations. (C) The antigenicity of VLPs was measured by two mAbs (capture: 13H12, detection: 3C3-HRP) using a sandwich ELISA.

13H12 was more sensitive than 3C3 to thimerosal treatment at intermediate thimerosal concentrations (i.e., 0.005% and 0.01% (*m/V*)) (Fig. 4B). In addition to the one-site binding assays, the relative antigenicity was also measured quantitatively by two mAb-based (capture: 13H12, detection: 3C3) sandwich ELISA assays. The EC_{50} values showed a 12-fold increase from 83 ± 1.1 ng/mL to 992 ± 1.1 ng/mL, when the VLPs were treated with thimerosal, indicating substantial weakening of the VLPs binding to these mAbs (Fig. 4C).

3.3.3. *The kinetics of epitope-specific antigenicity loss*

The sensor chip based SPR method was used to study the kinetics of HPV18 VLPs antigenicity alteration upon thimerosal treatment. SPR binding signals can be monitored in real time without need for labelling of either of the binding partners. Fig. 5A is a schematic diagram showing the detection principle of the SPR sensorgram using mAbs as molecular probes. The biphasic loss of antigenicity (normalized to the control at every time point) of the VLPs due to thimerosal treatment is compared to that of a control sample (left panel of Fig. 5B). The data were then fitted reasonably well to a double exponential decay model in the right panel of Fig. 5B with the parameters from the fitting tabulated in Table S2. The loss of antigenicity of VLP antigens occurred in a thimerosal concentration dependent manner (Fig. 5C). The relative antigenicity of 3C3 and 13H12 to VLPs by 0.01% (*m/V*) thimerosal treatment was reduced by approximately 70%–80% in the first 24 h. The antigenicity loss by SPR was also agreeable with the data obtained from the direct binding ELISA where the VLPs, with or without thimerosal treatment, were coated on the plate (Fig. 4B). As shown in Table S2, the majority of antigenicity loss occurred in the fast phase, with an $A_{fast}/(A_{fast} + A_{slow})$ ratio of 0.65 or higher for mAb 13H12 in the middle- and high-dose groups (≥0.005% (*m/V*)), while the ratio was observed for 3C3 in the low-dose groups (≤0.005% (*m/V*)). The k_{fast} of the antigenicity loss of VLPs to 13H12 was approximately 5–50 times higher in the lowest-dose group (0.001% (*m/V*)) than in the middle- or high-dose groups (≥0.005% (*m/V*)). For 3C3, the k_{fast} in the lowest-dose group (0.08 ± 0.007 min^{-1}) was only 3.0–3.5 times higher than that in the middle- or high-dose groups (Table S2). The k_{fast} values of both mAbs 13H12 and 3C3 were very close in the high-dose groups (≥0.01% (*m/V*)), which could indicate a large excess of ethylmercury, so the modification of the thiol (s) followed pseudo-first-order kinetics.

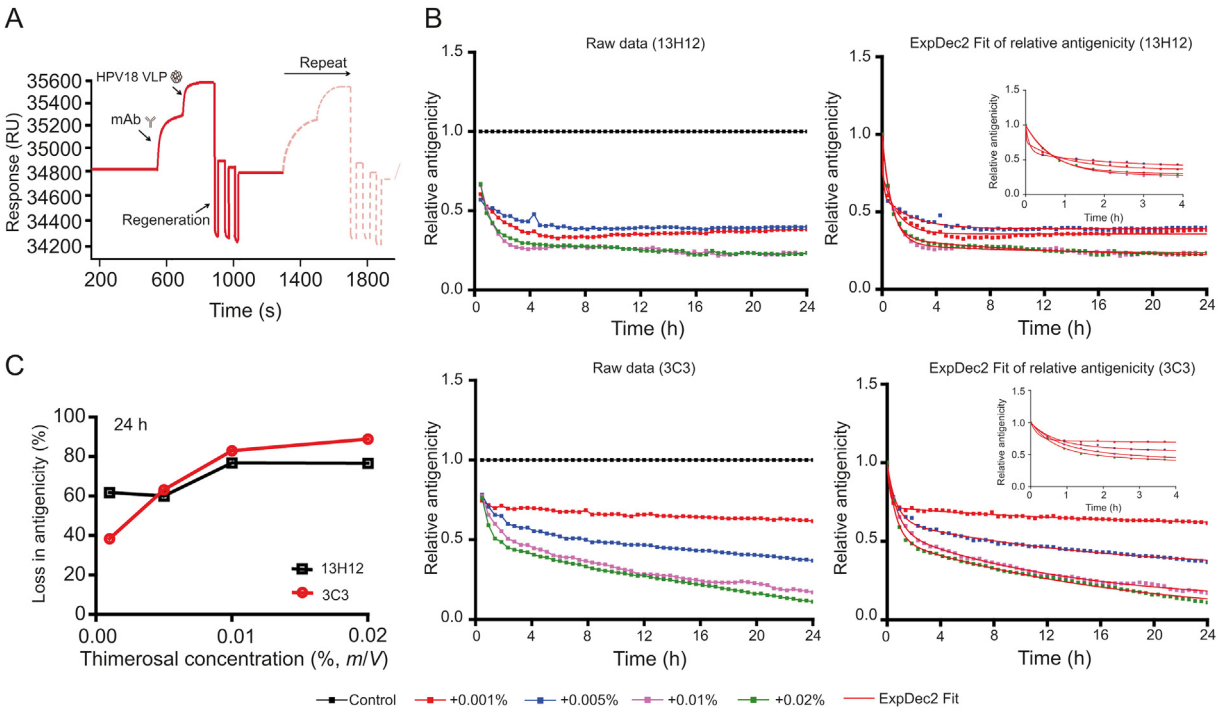

Fig. 5. The kinetics of HPV18 VLPs antigenicity loss as measured by SPR. (A) Schematic diagram showing the SPR measurement process. The binding activities of mAbs to the native and thimerosal-treated VLPs were measured at different times, and the binding cycles were repeated for 24 h. Each cycle for the binding activity yielded a data point. (B) In the left panel of Fig. 5B, the relative antigenicity of HPV18 VLPs with different concentrations of thimerosal treatment was measured by the mAbs 13H12 and 3C3. The kinetics of antigenicity loss upon thimerosal treatment was fitted to a double exponential equation, $F(t) = A_{fast} \cdot \exp(-k_{fast}\, t) + A_{slow} \cdot \exp(-k_{slow}\, t) + C$, and is shown in the right panel of Fig. 5B. In the inset, the first 4 h of the kinetics of antigenicity loss of VLPs is shown. (C) Dose-response relationship between the binding capacity and thimerosal concentration.

3.4. Visualization of the thimerosal-induced reduction in VLP antigenicity

As reported in the previous sections, the antigenicity in solution was measured by a one-site or two-site binding assay based on mAbs. Most vaccines are adjuvanted, containing the particle-adsorbed antigens in the formulation. In this part, an in situ method (HCA) was developed for visualizing and evaluating the antigenicity of VLP antigens adsorbed on micrometre-scale aluminium-based adjuvants in the presence of thimerosal. The distribution of fluorescence signals from the labelled mAbs on adjuvant particles with the adsorbed VLP antigen was clearly observed. The binding activity of mAbs 13H12 and 3C3 to the thimerosal-treated VLP antigen adsorbed on the adjuvant was shown to decrease according to the changes in fluorescence intensity owing to the labelled mAbs (Fig. 6A). The antigenicity of VLP antigens in the presence of thimerosal was expressed by the EC_{50} values, which was approximately 7- and 5-fold higher than their control groups for these two mAbs tested (Fig. 6B). The magnitude of change observed on the surface adsorbed antigen using these two mAbs separately was also in agreement with the antigenicity loss (approximately one order of magnitude) from the sandwich ELISA, where these two mAbs were used as capture (13H12) and detection (3C3) antibodies, respectively (Fig. 4C).

4. Discussion

In our previous studies, structural alterations were observed in HPV18 VLPs with thimerosal treatment via cryo-electron microscopy and three-dimensional reconstruction of the recombinant viral capsid. The reduction of immunogenicity of HPV16/18 due to thimerosal treatment was also demonstrated by the overall antibody or neutralizing antibody, as well as with epitope-specific competition. Covalent modifications of proteins by thimerosal may be the underlying mechanism for conformational changes that lead to reduced immunogenicity [15]. Among the four types of HPV studied (6, 11, 16 and 18), HPV 16 and 18 (type 18 in particular) showed the most prominent effects on structure and function due to the presence of the commonly used preservative thimerosal [15]. In the present study, we focused on capsid destabilization and antigenicity changes probed with a panel of murine mAbs of the recombinant HPV 18 viral capsid. Similarly, it has been reported that the epitope for a well-studied neutralizing mAb, mAb H16.V5 against HPV16 L1 VLP, was destroyed after incubation with thimerosal [14]. Like H16. V5 for HPV16, both 13H12 and 3C3 used in this study, are highly neutralizing mAbs for HPV18. These observations warrant more cautions on thimerosal use for protein based vaccines.

Therefore, the widely used vaccine preservative thimerosal may be not 'inert' with respect to the structure and function of the active protein components in drug or vaccine formulations. Recently, Strohmidel et al. [23] found the covalent binding between the $EtHg^{+}$ derived from thimerosal and haemagglutinin of a seasonal tetravalent influenza vaccine. The thiol-containing vaccine antigens are likely to be modified, as thimerosal is a specific thiol-capping or thiol-reactive agent. In this study, dramatic capsid destabilization of HPV18, probably due to thiol modification(s) at some accessible site(s), was demonstrated with the substantially reduced transition temperature in protein thermal unfolding by 25.3 °C and by 28.7 °C from the cloud point measurement with an enhanced propensity to aggregate upon thermal stress (Table 1 and Fig. 1C). In terms of function, a panel of mAbs showed varying degrees of alterations in binding activity to the HPV18 VLPs antigens upon thimerosal treatment. Specifically, the two mAbs (13H12 and 3C3) that showed

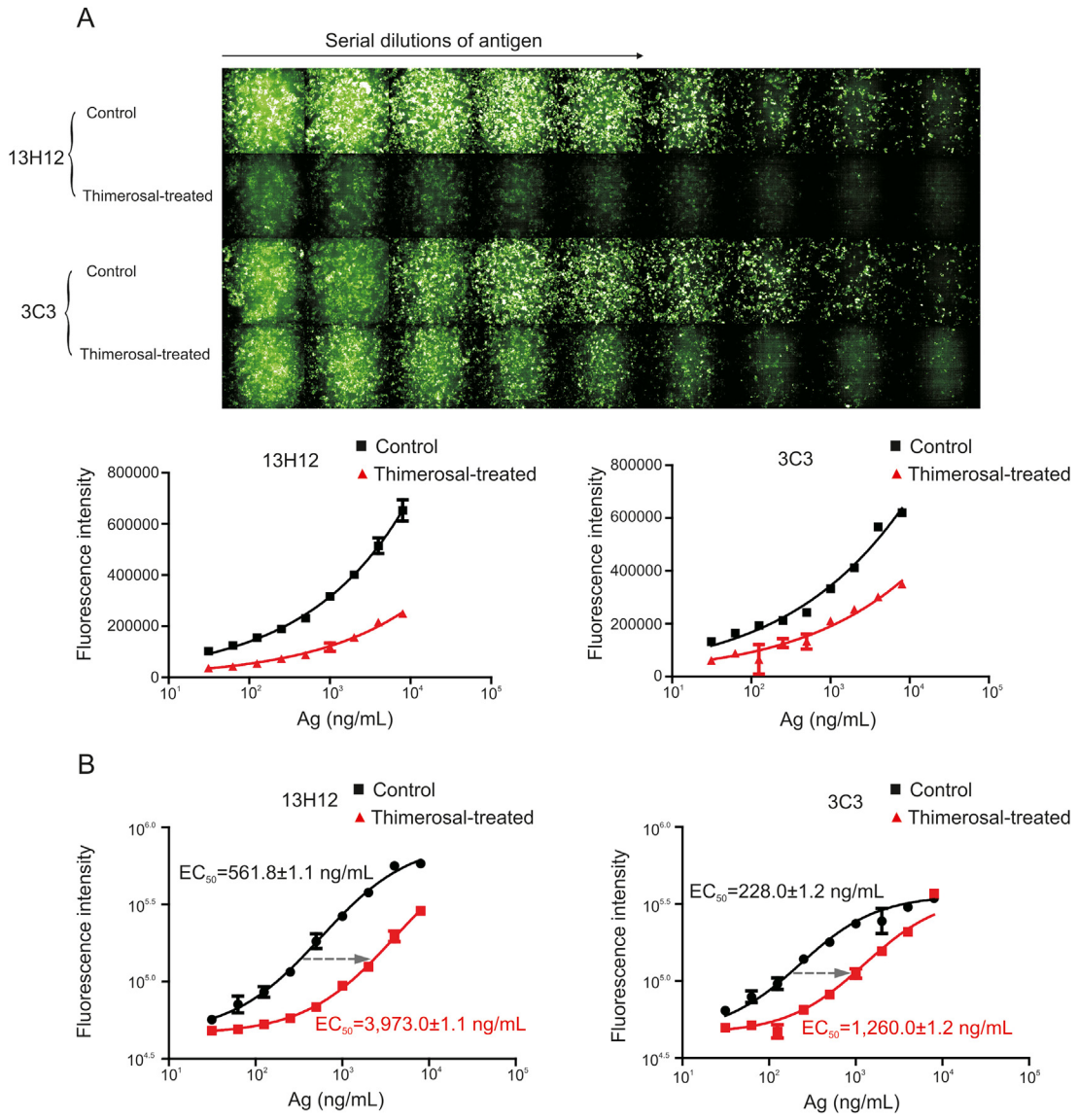

Fig. 6. Visualization of the VLP antigenicity decreases in the presence of thimerosal. (A) HCA images of the antigenicity of mAbs 13H12 and 3C3 to HPV18 VLPs adsorbed on aluminium adjuvants in the presence of thimerosal or absence of thimerosal. The intensity of fluorescence in only part of the whole visual field was read by the Opera Phenix high content system. (B) The antigenicity of VLP antigens with or without thimerosal treatment was analysed by a fluorescence plate reader. The EC_{50} means the concentration of antigen required to achieve 50% of the maximal signal.

the highest sensitivity to thimerosal treatment are also type-specific with high virus neutralizing efficiency. The kinetic differences in the antigenicity loss when monitored with these two distinctly different mAbs (13H12 and 3C3) would indicate that the loss of immunoreactivity is epitope-specific. Both the ELISA (measuring the immunocomplexes at a given equilibrium) and the SPR-based kinetic binding assay (measured in real time with no wash steps) yielded similar conclusions for the thimerosal concentration dependence and the epitope-specific manner of the antigenicity changes.

The phenomenon of epitope alterations in the presence of thimerosal has been reported for other vaccines. Kraan et al. [11] reported that the presence of thimerosal resulted in a temperature-dependent loss of polio D-antigen by D-antigen ELISA using a mixture of serotype-specific anti-poliovirus mAbs. D-antigen could be recovered by addition of L-cysteine into the thimerosal-containing vaccine formulation, probably through the regeneration of some critical thiol group(s) by the incoming free thiols in the form of overwhelming amount of cysteine. Similarly, a significant reduction was observed in its immunoreactivity by using a specific mAb-based assay for a thimerosal-containing formulation of glycoprotein 63 from *Leishmania* after one year of storage at 4 °C [24]. The findings presented by Harmsen et al. [25] showed that thimerosal could stimulate intact (146S) foot-and-mouth disease virions to dissociate into 12S particles when monitored with mAb-based ELISA. Such dissociation could result in strongly reduced immunogenicity.

Although alterations in the antigenicity and immunogenicity of vaccine antigens had been observed, the in-depth characterization of the kinetics of the thiol modification of antigens was not previously available. The kinetics of epitope modification could vary because the reactivity and accessibility of the thiol groups could differ significantly. It has been reported that the antigenicity of poliovirus type 2 was unchanged when ELISA was performed with monoclonal 9Ab after thimerosal incubation for 1 h at 25 °C. However, the other mAb (monoclonal 7Ab) based potency of

poliovirus type 2 was completely lost within 5 min of incubation with thimerosal at 25 °C or 37 °C [10]. In this study, the SPR data showed that the half-life of the relative antigenicity loss for either 13H12 or 3C3 was approximately 6 or 9 h based on the k_{slow} at 25 °C upon thimerosal treatment. If the HPV vaccine formulation contained 40 μg of HPV18 L1 proteins in a 0.5 mL dose containing 0.01% thimerosal, the mole ratio of Hg^{+} from thimerosal to cysteines (14) in the HPV18 L1 proteins was 11 (0.125 μmol: 0.0112 μmol). If only one cysteine is accessible for forming a covalent bond with an incoming $EtHg^{+}$, the molar ratio would be 150. The presence of an overwhelming excess of ethylmercury compared to the target thiol group(s) also explained the good fits using the biphasic pseudo-first-order kinetics model for the antigenicity loss data obtained after monitoring with two mAbs (Fig. 5B and Table S2). In addition, it has been reported that the mercury(II) tightly bonds to the histidine imidazole tightly and the k_{fast} (13.28 ± 0.07 min^{-1}) is approximately one or two orders of magnitude higher than the k_{fast} from SPR data (0.02–1.2 min^{-1}) [26]. The kinetics results of antigenicity loss should rule out the binding to the imidazole of histidine residues to be the basis for immunoreactivity change, as this would be a much faster event. Thiol modification is likely to be the underlying chemical events, causing impairments in clinically relevant epitopes.

Thimerosal can spontaneously degrade into thiosalicylic acid and ethylmercury in aqueous media. The strong affinity of mercuric ions (Hg^{2+}) and their alkyl derivatives towards sulfhydryl groups (-SH) in amino acids, peptides and proteins is well documented [27–29]. An in vivo study in humans performed by Trümpler et al. [30] reported the observation of ethylmercury-glutathione adducts. They also investigated the interaction between β-lactoglobulin A and thimerosal in simulated physiological conditions (pH 7.4, at 37 °C) for 1 h, and the results showed that a free thiol residue in peptide T13 (amino acids 104–124) is the binding site of ethylmercury by means of LC/ESI-TOF-MS and LC/ICP-MS [31]. Hogeback et al. [32] studied the adduct formation of organic mercury species with carbonic anhydrase and heamoglobin, and found that the free cysteine residues within proteins are the binding sites of methylmercury and ethylmercury cations. Janzen et al. [33] also showed that the binding stoichiometry correlates with the number of free thiols in the α- and β-chains of heamoglobin. In addition, thimerosal forms a bovine serum albumin-ethylmercury adduct with thiosalicylic acid release through the free Cys 34 residue and changes the conformation of bovine serum albumin [34]. In another study, Ishii et al. [35] found that HPV16 pseudovirions lose their infectivity to HeLa cells after incubation with thiol-reactive reagents. It has been demonstrated that HPV16 L1 protein-free thiols in C146, C225 and C229 are accessible to thiol reactive agents by mass spectrometry and mutational analysis. The capping of these thiols or ethylmercury-cysteine adduct formation might result in reduced infectivity [35]. Since sulfhydryl modifications via covalent bonding should be responsible for the epitope alteration of the HPV18 viral capsid, further efforts are needed to identify the specific cysteine residue(s) involved in the thiol modification of HPV18 VLPs antigens in the presence of thimerosal.

Currently, although thimerosal-free single-dose vials and peadiatric vaccines are required, a preservative is still necessary in multi-dose vials of influenza vaccines (Table S1). Therefore, choosing thimerosal as a preservative for future use needs to be carefully studied based on its molecular structure, especially for thiol-containing proteins in vaccines and other biological products. In-depth characterization should be performed on the potential impact to the antigen structures and, even more importantly, its functions such as antigenicity and immunogenicity. Recently, Agarwal et al. [36] found that thimerosal destabilizes aluminium-based adjuvant adsorbed recombinant subunit rotavirus vaccine antigens and induces loss in immunoreactivity as reflected by mAb-based ELISA binding ability. If thimerosal is used in bioprocessing procedures, free cysteine could be used later in the process for the regeneration of the critical cysteine residues, reversing some of the effects incurred due to the presence of thimerosal. In addition, alternative preservatives, such as 2-phenoxyethanol, which have been used in pneumococcal multi-dose vaccine formulations [37], should be considered for use in vaccine formulations when thimerosal is incompatible with the target antigens.

5. Conclusions

This study significantly advanced our understanding on the kinetics and nature of thiol-modifications by thimerosal, leading to partial or even complete loss of antigen function. Epitope-specific alterations in the antigenicity of HPV18 VLPs were characterized using a set of mAb-based immunochemical assays after thimerosal exposure. Deleterious effects of thiol-reactive thimerosal on the antigenicity and protein conformational stability of HPV18 VLPs were demonstrated in a thimerosal concentration-dependent manner with the time scale of antigen modifications defined. The kinetics of antigenicity loss due to thimerosal treatment was monitored with two different mAbs, showing distinctly different sets of kinetic parameters. Thiol modification may be the underlying event that results in the loss of antigenicity of HPV18 VLPs, coupled with structural alterations and substantially reduced protein stability. Efforts should be made in an in-depth understanding of the process and formulation with respect to the presence and maintenance of the clinically relevant epitopes on the vaccine antigen during vaccine bioprocessing, formulation and transportation.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2020.08.007.

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