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The differential effect of sub-micron level HA aggregates on influenza potency assays

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ABSTRACT

Influenza vaccines remain the most effective public health measure for the prevention of influenza-related illnesses. The primary immunogen in inactivated influenza vaccines is hemagglutinin (HA), the receptor binding protein of influenza. The concentration of HA during vaccine production and testing is standardized according to the level of antigen as measured by Single Radial Immunodiffusion Assay (SRID). This allows vaccine potency to be controlled such that individuals receive a dose known to provoke a clinically protective immune response. As compared to alternatives, SRID has the advantage of quantifying immunologically relevant forms of HA, but it depends on timely generation of novel reagents for each new vaccine strain. In recent years, a number of alternative assays have been suggested based on either epitope recognition, receptor binding or protection from proteolysis but it is unclear how they relate to vaccine potency in clinical trials. In this report we describe the development of a lectin-based, ELISA-type assay for HA potency and find it provides similar potency estimates to SRID except in the case of a vaccine with aggregated HA and other viral proteins. In that case, SRID predicted the immunologically active HA present and ELISA techniques did not. This difference was due to tested antibodies failing to pull down or bind to the HA present unless particle aggregates were first dissociated. Furthermore, detergent treatment alone was insufficient to complete this dissociation. While others have previously demonstrated that immunocapture-based techniques can misestimate the potency of influenza vaccines depending on the individual antibodies used we demonstrate that in this case the failure was due to an inability of all antibodies to capture HA contained in the aggregated influenza vaccine.

1. Introduction

Influenza viruses infect up to 20% of the population in a typical season resulting in a sharp rise in the number of medical clinic and hospital visits as well as an elevation in seasonal mortality rates [1]. Annual epidemics, the result of antigenic drift in the circulating strain or sub-type, predominately affect the elderly, immunosuppressed and other high risk groups. Periodic pandemics, the result of an immunologically novel influenza strain with capacity for sustained transmission in humans, can result in a significant rise in morbidity and mortality amongst healthy children and adults [1]. Currently, the best protective public health measure against infection is immunisation with a vaccine well-matched to the circulating strain(s) [2].

The potency of influenza vaccines is currently measured through the single radial immunodiffusion SRID assay. The SRID is an agarose gel based assay in which antibodies raised against the specific strain of HA to be tested are embedded in the gel matrix. Subsequent interaction with HA containing samples result in precipitation of these complexes over time enabling quantification of the HA present. Since its implementation in 1978 as the standard tool for determining influenza vaccine potency the SRID assay has proven to be robust, simple and adaptable to the increasing number of vaccine strains contained in a seasonal influenza vaccine [3]. Despite this, there are disadvantages of the SRID assay. Namely it is labour intensive, it is dependent on the timely production of novel reagents (antisera and reference standards) and it is not capable of measuring very low quantities of antigen nor vaccines containing many common adjuvants [4]. For these reasons there has been a push to identify and evaluate alternative assays for the determination of potency in influenza viruses [5,6].

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A number of methods including quantification through SDS-PAGE, slot-blot, ELISA, Mass Spectrometry and HPLC have been published [7–11]. These initial alternative assays were not capable of distinguishing different antigenic structures of the influenza hemagglutinin (HA) molecule and thus could result in an over-estimation of the quantity of immunogenic HA. To resolve this, different groups have suggested and applied filtration or gating techniques based on biological activity, such as binding to recombinant receptors, immunocapture techniques using conformationally sensitive antibodies or proteolysis which specifically targets non-trimeric forms of HA [12–17]. Additionally several papers reported variation in potency determined by immunocapture techniques based on the antibody properties used [17,18].

The initial goal of this study was to evaluate how a lectin-based binding or detection platform could simultaneously measure both HA and NA levels in an inactivated influenza vaccine. Lectins are carbohydrate-binding proteins that promote cell agglutination with properties similar to antibodies, with broader cross-reactivity [19]. Lectins serve in many cellular targeting and communication processes and are expressed in plants, animals and the pathogens that target them [20]. Various plant lectins have been used in biotechnology applications including blood typing, virus isolation or enrichment, glycan detection/isolation, protein induction, diagnostic and/or therapeutic agents [21].

In this report we demonstrate the use of *Crotalaria juncea* lectin, a lectin derived from sunn hemp seeds, as a universal capture reagent for the only glycosylated proteins in influenza vaccines HA and NA. The bound influenza proteins can then be quantified with specific antibodies. The lectin-based assay accurately quantifies HA in monovalent vaccine samples. However, when used to test an array of trivalent vaccines, several clinically effective products appeared to contain very low levels of HA. We confirmed that there were no changes to the expected protein sequence or glycosylation levels in the vaccines. However, the H1 HA antigen in these vaccines were not significantly bound by any antibodies tested when evaluated in solution. This observation correlated with significant levels of submicron-sized aggregates contained within these vaccines. These HA aggregates were dissociable using heat and detergent, and after treatment, some antigenic recognition was restored. This suggests that the formation of submicron aggregates comprised of native, antigenic trimers will interfere with the detection of HA in assays that rely on antibody-binding or capture in a liquid environment for the quantification of immunogenic HA.

2. Materials and methods

2.1. Vaccines

Monovalent split inactivated A/California/7/2009 (H1N1) or A/turkey/Turkey/1/2005 (H5N1) and Trivalent split inactivated vaccines containing A/California/7/2009, A/Texas/50/2012 and B/ B/Massachusets/2/2012 strains were obtained from vaccine manufacturers. All vaccines were produced in eggs and all standards used were from the National Institute for Biological Standards and Control NIBSC.

2.2. Antibodies, receptors and chemicals

Mouse antibody C179 was acquired through TakaraBio (Takara Bio, Mountain View, CA USA), Rabbit H1N1 (RM01 & RP03) A/California/7/2009 antibodies were acquired through Sinobiogicals (Sinobiological, PA, USA) and Sheep anti-A/California/7/2009 (16/144) was acquired from NIBSC (NIBSC, UK). *Crotalaria juncea* lectin was acquired through Medicago (Medicago, AB Denmark). Proteolysis was completed using 100 μg/ml (TPCK)-treated trypsin (New England Biolabs) for 30 min at 20 °C.

2.3. Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA was performed as previously described [14]. Briefly, 96-well plates (Nunc, Canada) were coated overnight at 4 °C with 100 μL 10 μg/mL of *Crotalaria juncea* in 0.05 M carbonate buffer (pH 9.6) per well. All washing steps were conducted with PBS containing 0.05% (v/v) Tween-20 (PBS-T), blocking was done with 5% (w/v) skim milk in PBS-T for 1 h at 37 °C. Vaccine or references standards were detected with A/California/7/2009 strain specific antibodies (C179 or RP01). Peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Sigma-Aldrich Canada Ltd., ON) was used to detect bound antibodies. TMB substrate (New England Biolabs, Ltd.ON, Canada) was added for colorimetric development. Vaccine concentrations were determined by sigmoidal curve fitting with Combistats 5.0 software (Council of Europe, EDQM).

2.4. Single-radial-immunodiffusion Assay (SRID)

HA content was determined using the SRID assay as previously described [22]. Vaccine and control samples were treated for 20 min at room temperature with Zwittergent 3–14 (Calbiochem-Behring Corp., La Jolla, CA) at a final concentration of 1% (w/v). Vaccines or reference standards were diluted, loaded and incubated for 24 h at room temperature. Following incubation, the gels were washed mounted onto Gelbond film (Lonza, Rockland, ME, USA), dried and stained with Coomassie Brilliant Blue. Vaccine concentrations were determined by parallel line analysis using Combistats 5.0 software (Council of Europe EDQM).

2.5. SDS-PAGE and western blots

Samples for SDS-PAGE and western blot analysis were boiled in 4X Laemmli sample buffer either with (reducing) or without (non-reducing) beta-mercaptoethanol. Proteins were separated by running the samples on Bio-Rad Mini-protean TGX Stain-free precast gels 4–15%, 10 wells. Total protein load was visualized by activating the gels for 1 min under UV conditions as per manufacturers recommendations (Bio-Rad Laboratories (Canada) Ltd. ON, Canada). For Western blots the separated proteins were transferred to polyvinylidene fluoride (PVDF) (MilliporeSigma Canada Oakville, ON) and HA proteins were visualized by blotting with a 1:3448 dilution of anti-HA Rabbit polyclonal (Sinobiological Inc. RP03) and a 1:75,000 dilution with horseradish peroxidase linked anti-rabbit goat antibodies (Thermo Scientific Canada). Detection was with ECL reagents (Thermo Scientific Canada). Mississauga, ON) for 20 min.

2.6. Mouse immunogenicity study and serological analysis

Adult male C57/BL6 mice (Charles River Laboratories, Montreal, QC) from 6 to 8 weeks old were used in all experiments. Animals were housed in autoclaved cages in a pathogen-free environment for 1 week prior to use. Mice were provided access to autoclaved food (Purina Lab Chow #5001) and water. All experiments were conducted according to CCAC guidelines.

To assess possible differences between the two vaccines in terms of immunogenicity mice were immunized once with one of the two different trivalent formulations. Mice were divided into groups of 10 with each mouse receiving the indicated dose s.c.. Twenty-eight days post-vaccinations sera was collected from vaccinated and unvaccinated animals.

Initially all sera (pre-and post-vaccination) were mixed 1:3 overnight with RDE (Denka Seiken, Tokyo, Japan) and heat inactivated at 56 °C for 30 min and then diluted to a final concentration of 1:10 with PBS. 2-fold serial dilutions of 25 μL of sera were pre-incubated with 4 HA units of A/California/7/2009 antigen (NIBSC) for 30 min at room temperature. 50 μL of 0.5% chicken RBC were added and the plate was incubated for 30 min at RT. Wells were examined visually and compared to control wells with RBC + PBS or RBC + PBS + HA antigen. HAI titers were the reciprocal of the highest dilution of RDE-treated serum that inhibited hemagglutination of the RBCs. A potentially protective response was set to be equivalent to an HI titer of 40 or greater as is generally agreed upon in human immunogenicity studies [23]. While there isn't a similar documented correlation between neutralizing titer and murine protection from infection the same HI titer was used for consistency. All HI titers <20 (detection limit) were arbitrarily set at a value of 10. Results were tabulated with Graphpad Prism 6 (GraphPad Software Inc., San Diego) and titers were transformed by the equation $Y = 1/-1 * \text{Log}(Y)$. Statistical analysis of these transformed results was conducted with a two-tailed *t*-test for unpaired samples and normal distribution was assumed. Those groups which displayed a P value <0.05 were judged to be significant

2.7. Immunoprecipitation and RP-HPLC

Briefly 100 μL of vaccines was incubated with either 20 μg/ml of purified C179 or RP03 antibody or a 1:2000 dilution of purified sheep anti-sera. Antibodies and vaccines were rocked overnight at 4 °C before being incubated with Peirce Protein A/G Surebeads (Thermo Fisher Scientific, Canada) for a further 2 h at 4 °C. After washing steps antibody-antigen complexes were removed from beads with 1% w/v 3–14 Zwittergent (Calbiochem-Behring Corp., La Jolla, CA) and both elute (bound fraction) and the reserved supernatant (unbound fraction) were analyzed by RP-HPLC.

The analytical HPLC system consisted of a Waters Alliance 2695 chromatograph equipped with a column heater and an auto-sampler with a sample cooling device coupled to a Waters 2475 Multichannel Fluorescence Detector with a 8 μL flow cell working at λ_{ex} 280 nm and λ_{em} 335 nm and a Waters 2996 UV–VIS photodiode array detector (Waters, QC, Canada). Data acquisition and integration were performed with Empower 3 Chromatography Data Software from Waters. Separation of HA vaccine samples was obtained using previously reported methods [24,25]. Briefly, chromatographic separations were carried out at 55 °C with an AB gradient elution of 19 min at a flow rate of 1.0 mL/min where composition of eluent A was 0.04% (v/v) aqueous TFA and eluent B was 0.03% (v/v) TFA in 25% ACN and 75% 2-propanol [25].

2.8. Nanoparticle tracking analysis

Vaccine samples were left untreated or raised to indicated temperatures for the indicated times in the presence or absence of 1% w/v zwittergent 3–14 (Calbiochem-Behring Corp., La Jolla, CA) before being analyzed on the Nanosight NS 300 (Malvern Instruments Ltd, Worcestershire, UK) as previously described [26]. The Nanosight was used according to the manufacturer's instructions with appropriate camera levels and flow rates. Reproducibility of counting was determined using 3K-150 Series Particle Counter Standards (152 ± 5 nm, ThermoFisher Scientific, Waltham, Ma, USA). Distribution widths were expressed as:

$$Span = \frac{Dv90 - Dv10}{Dv50}$$

Where 90 percent of the distribution lies below the *Dv90* and 10 percent of the population lies below the *Dv10* while *Dv 50* is the median.

3. Results

3.1. Crotalaria juncea lectin as capture reagent for ELISA

To develop this influenza potency assay, we selected a tetrameric lectin from *Crotaluria juncea* (sunn hemp), which binds strongly to β-galactosides, including both HA and NA [27]. We paired this lectin with the anti-HA antibody C179, a universal-type "stalk" antibody that neutralizes all group 1 influenza A viruses that have been tested [28]. The *Crotaluria juncea* lectin and C179 combination detected an initial panel of group 1 (H1, H2, H5) influenza antigens, and none of the tested group 2 (H3) or B antigens (Fig. 1A). In spiking and recovery assays, this same ELISA technique was sensitive to a dose response of the quantity of H1 or H5 antigen at 3 concentrations (80%, 100%, and 120%) (Fig. 1B and Table 1).

3.2. Potency of H1 in monovalent and trivalent vaccines

As demonstrated in Fig. 2, the ELISA was also able to quantify HA present in H1 and H5 monovalent vaccines, similar to SRID. This method detects the loss of trimeric HA antigenicity in monovalent vaccine preparations after heating to elevated temperatures, corresponding to the results observed by SRID (Fig. 2, Table 2). However, in testing trivalent vaccine preparations, which are used in clinical seasonal vaccines, not all results were similar between the ELISA and SRID methods (Table 2). Similar to our results when testing the monovalent vaccines, the H1 HA from vaccine A was detected and quantified similar to SRID although levels detected were always slightly higher. The H1 HA from vaccine B was not readily measurable under any tested conditions by ELISA (Table 2). This was not attributable to any detectable difference in protein sequence, quantity or modification as evaluated through tryptic digestion followed by liquid chromatography/mass spectrometry (LC/MS-MS) (Supplementary Figs. 1 and 2) while immunogenicity experiments in mice indicated the vaccines generated an H1 antibody response that was not significantly different from each other (Fig. 3).

3.3. Characterization of vaccines by SDS-PAGE and western blots

We next compared the total protein and specific H1 HA content contained in the vaccines by SDS-PAGE gels and Western blots. On reducing gels, vaccine A displays intense bands running at levels consistent with HA1, HA2 NA and M1 in addition to fainter bands primarily under 50 kDa as well as a faint band at approximately 75 kDa. While vaccine B displays similar sized bands however it also contains distinct bands at 75 and 100 kDa as well as a series of dark staining bands above 100 kDa (Fig. 4A). Significant differences between the vaccines were also observed on non-reducing gels (Fig. 4B). Vaccine A displays 4 primary bands with the strongest at 75 kDa, corresponding in size to unreduced HA monomers as well as a higher band running slightly above 150 kDa. Trivalent vaccine B contains a greater number of bands at higher molecular weights and of greater intensity than observed in A with bands corresponding to HA dimers and trimers and greater molecular weight. We next probed the conformation and thermal sensitivity of the HA contained in vaccines A and B by treating vaccines with different combinations of trypsin and thermal stress prior to separating proteins through reduced SDS-PAGE gel and analyzed HA through transfer to PVDF membrane probed with antibodies

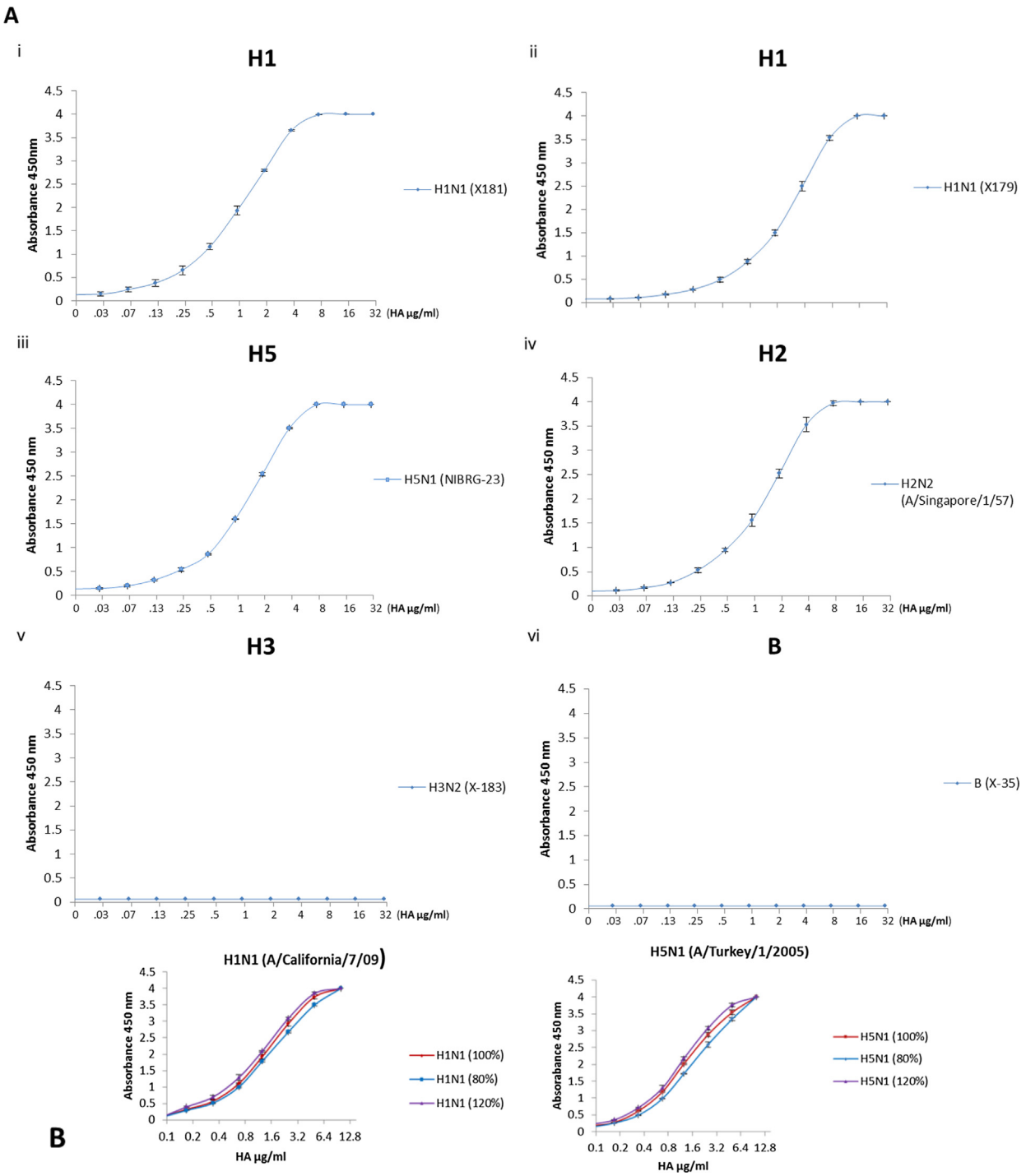

Fig. 1. Specificity and precision: Detection of bound Influenza Antigens by C179. (A) *Crotalaria juncea* lectin was used to coat plates at a concentration of 10 µg/ml. Vaccine Antigens (i) A/California/7/2009 (NYMC-X181), (ii) A/California/7/2009 (NYMC- X179), (iii) A/turkey/Turkey/1/2005 (NIBRG-23), (iv) A/Singapore/1/57(v) A/Wisonsin/15/2009 (NYMC-X183) (vi) B/Brisbane/60/2008 (NYMC-BX35) were diluted and applied to lectin coated plate prior to MAb C179 (Takara) being applied at 2 µg/ml for one hour. **(B)** Three ratios, 80%, 100% and 120% of HA from H1N1 (i) (A/California/7/2009 NYMC-X181) and H5N1 (ii) (A/turkey/Turkey/1/2005 NIB-23a) reference antigens. Reference antigens were diluted to 10 µg/ml and were used in spiking and recovery assays. Experiments were repeated in tripicate and data are shown as the mean ± SD. Potency values were determined as described in materials and methods and values are reported in Table 2.

specific for A/California/7/2009 HA (Fig. 4C). Vaccine A displays similar staining pattern to the H1 standard with two clear bands corresponding to HA1 and HA2. These bands show resistance to trypsin degradation (lane 4) indicating that they are present in the native pre-fusogenic trimer conformation [12,29]. Pre-treatment for an hour at 55 °C exposes the trypsin cleavage sites contained in HA1, consistent with the typical pattern observed for HA from the A/California/7/2009 strains [22]. In vaccine B there are 3 distinct bands which correspond to HA1 and HA2 as well as either monomeric HA or HA0, in addition to some fainter bands of greater molecular weight. Treatment with trypsin does little to change this pattern, demonstrating that the band of 75 kDa is likely monomeric HA derived from a pre-fusogenic HA trimer. Holding this vaccine at 55 °C partially exposes the tryptic cleavage

Table 1
Spiking and Recover. HA values were adjusted to 10 ug/ml and compared to different input HA amounts to measure changes in concentration. Each experiment measured values in triplicate and data are shown as ±SD from two separate experiments.

Observed concentration	Expected concentration	Recovery
A/California/4/2009		
9.7 (9.5–10.0)	10	97.2 (94.7–99.8)
8.6 (8.2–9.0)	8	107.1 (102.3–112.2)
11.5 (10.9–12.0)	12	95.4 (91.2–99.9)
A/Turkey/1/2005		
10.1 (9.6–10.7)	10	101.4 (95.9–107.4)
8.0 (7.7–8.4)	8	100.6 (96.3–105.2)
11.6 (11.1–12.1)	12	96.7 (92.5–101.0)

Table 2
Quantification of vaccines. Paired vaccine sanmples were quaitified by both ELISA and SRID and the values compared. Each sample was tested in triplicate and values shown are the mean (95% cofidence interval).

Vaccine	SRID μg/ml	ELISA μg/ml
H1N1 MV	14.2 (13.8–14.6)	13.9 (13.3–14.5)
H1N1 MV 45 °C 1 h	7.7 (3.2–11.4)	5.2 (4.8–5.5)
H1N1 MV 50 °C 1 h	ND	2.1 (1.9–2.3)
H5N1 MV	15.54 (14.1–16.8)	17.9 (16.8–19)
H5N1 MV 50 °C 1 h	12.2 (10.7–13.6)	11.9 (11.2–12.7)
H5N1 MV 60 °C 1 h	2.3 (0–7.6)	3.5 (3.3–3.8)
Vaccine A Lot 1	26.6 (23.9–29.3)	33.4 (27.3–38.1)
Vaccine A Lot 2	27.7 (24.5–30.4)	32.7 (30.9–36.5)
Vaccine A Lot 3	27.5 (25.7–29.4)	36.1 (31.5–41.3)
Vaccine B Lot 1	46.4 (42.4–50.3)	1.8 (0.5–3.9)
Vaccine B Lot 2	46.5 (42.7–51.0)	1.2 (0.6–2.9)
Vaccine B Lot 3	45.2 (43.3–48.7)	1.5 (0.5–3.8)

sites although do we begin to see the presence of breakdown products indicating that some degradation is occurring in addition to a qualitative decrease in the staining density of the higher order HA bands (Fig. 4C). To confirm that this correlates with the thermal protection of the antigenic HA we compared the antigenicity of these vaccines pre- and post-thermal treatment using SRID analysis (Fig. 4D). While both vaccines are after both vaccines are treated at 55 °C there is some retention antigenic HA in vaccine B while none remains in vaccine A. All antigenicity is lost from both vaccines after treatment at 80 °C. The overall pattern of vaccine B in both the SDS-PAGE gels and western blots is consistent with H1 HA and possibly other proteins being aggregated. These aggregates appear to comprise native HA trimers and to correlate with an increase in the thermal stability of the HA trimer.

3.4. Immunoprecipitation of H1 from trivalent vaccines

To test the antibody detection element of the lectin-based ELISA in isolation from other immunological or binding agents we used C179 to immunoprecipitate the H1 fraction of these two vaccines. The HA fractions, both precipitated and not, were detected by RP-HPLC as previously demonstrated [24]. As can be clearly seen in Fig. 5A, the H1 component of vaccine A is immunoprecipitated with C179, while the H1 from vaccine B is not specifically captured. In order to evaluate if this result was due to a specific epitope being blocked we tested several other antibodies. The results were seemingly identical; when RP03 (rabbit polyclonal specific for A/California/7/2009) and 16/144 (sheep anti-sera against A/California/7/2009) were used the antibodies could interact with and precipitate the H1 fraction from vaccine A but not vaccine B.

To test if a component of the vaccine buffer was interfering with antibody binding to HA we inactivated the HA contained in one vaccine and mixed it with the second vaccine. When inactivated vaccine B was added to vaccine A, the H1 signal was similar to vaccine A + PBS, and conversely when inactivated vaccine A was added to vaccine B, HA was detected similarly to vaccine B + PBS. This rules out any influence of the vaccine formulations in promoting or inhibiting the binding of the antibodies to the HA contained in these vaccines (Fig. 5B).

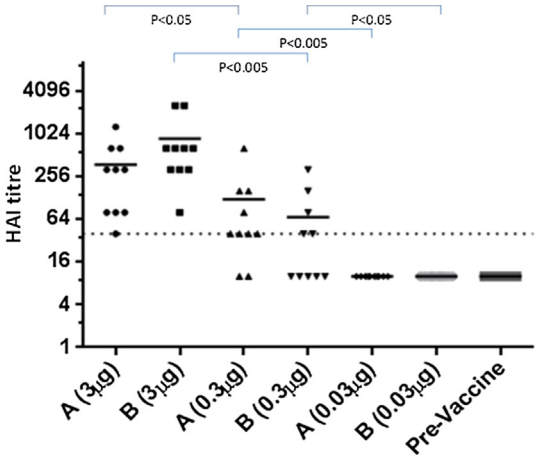

Fig. 3. Hemagglutination inhibition results from Antigen-specific serum antibody responses: Groups of ten mice were adminsitered 50 μL of vaccine preparation at two different sites to achieve a total administered vaccine as indicated. 28 days later serum was withdrawn from the individual mice. Specific A/California/7/2009 antibody titers were determined for pre and post-vaccination serum by hemagglutination inhibition assay as detailed in the Materials and Methods. Any results below a HAI titre of 20 were reported as 10. Experimental mean is indicated by a line and the individual titers measured are indicated. *P-values* below 0.05 were observed between dosage groups but were not observed between different vaccines at a matched dosage level.

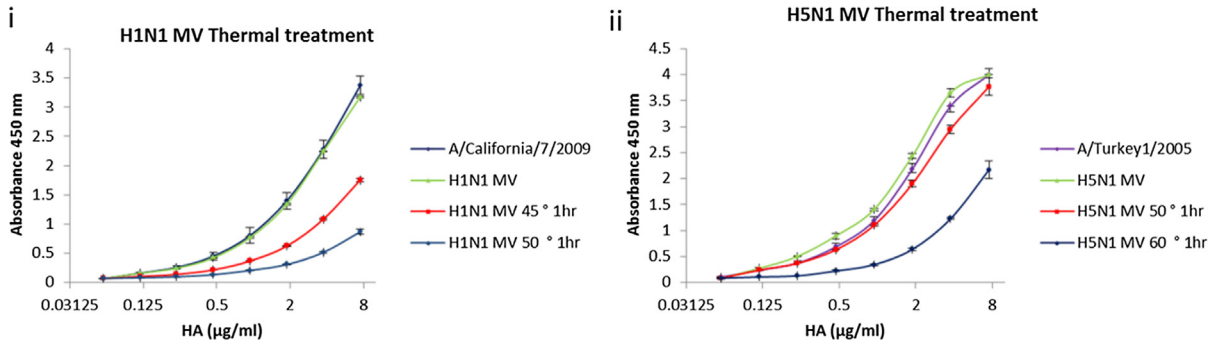

Fig. 2. Effect of heat treatment on vaccine potency. Influenza monovalent H1N1 (i) or H5N1 (ii) vaccines were incubated held at 4 °C or elevated to temperatures indicated after which HA potency was determined by the Lectin-based ELISA using the Group 1-binding Mab C179 (Takara). Experiments were repeated in triplicate and the results are reported as the mean of the absorbance detected ± SD. Potency values were determined as described in materials and methods and values are reported in Table 2.

3.5. Particle sizing

Nanoparticle Tracking Analysis (NTA) allow particle visualization and tracking which means that the particle sizes (above 80 nm) and their numbers which make up the lipid/protein fraction of a biological product can be measured [30] This visualization and tracking is an accepted method for determining the relative overall aggregation levels contained in a vaccine in the

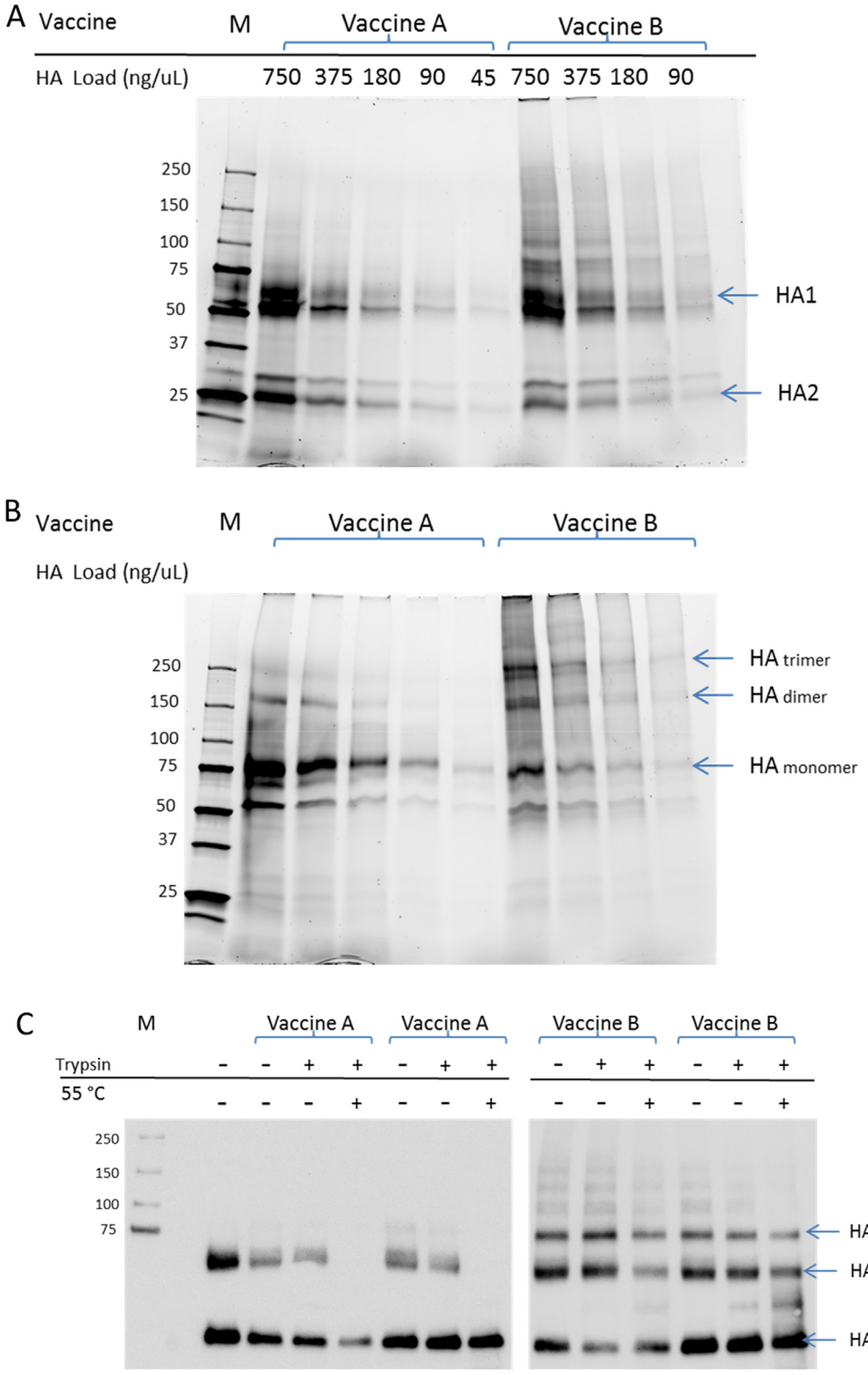

Fig. 4. Comparison of trivalent vaccines through SDS-PAGE, western blot and SRID analysis. Vaccines were run on both reducing and non-reducing gels to compare the overall protein load and behaviour between the two trivalent vacines. On the reduced **(A)** and non-reduced **(B)** gels vaccines were loaded in a serial dilution as indicated. Bands corresponding to the expected sizes of HA1 and HA2 **(A)** or HA monomers, dimers and trimers are indicated **(B)**. **(C)** Vaccines treated as indicated before being run on reducing gels and analyzed by western blot. HA was detected with a Rabbit polyclonal antibody RP03, specific for A/California/7/2009 strains. HA monomers, as well as HA1 ad HA2 are indicated. **(D)** Vaccines were treated at the indicated temperature for one hour before being loaded onto SRID gels for analysis.

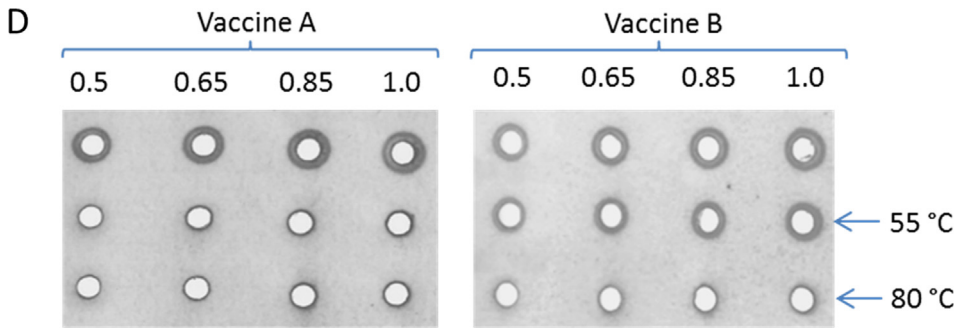

Fig. 4 (*continued*)

sub-micron level [31]. Particle sizes and numbers contained in the two trivalent vaccines were compared through triplicate runs of different lots of each and the results are displayed in Fig. 6i-iv and Table 3. Trivalent vaccine A was comprised of a single peak (mode 132 nm) with an extended shoulder [32]. Small differences were observed around 180–220 nm in samples heated to 37 °C which may reflect some increase in protein aggregation at this temperature (Fig. 6i and Table 3) but these differences are not retained at 55 °C. Overall, vaccine A demonstrated the expected distribution for largely unaggregated particles (Fig. 6i) [33]. Vaccine B on the other hand, has three distinct peaks, the most abundant at 131 nm with a second at 215 nm and third at about 380 nm suggesting three populations of particles all in the submicron range (Fig. 6iii and Table 3). Initial heating of the sample to 37 °C resulted in fewer particles in the larger peaks as well as a reduction in the average size of particle forming these peaks. Heating vaccine B to 55 °C continued this trend with more particles in the sub-200 nm range. After incubation with 1% w/v zwittergent 3–14 for 60 min, vaccine A maintained its distribution pattern, with an overall decrease in the size of the particles (Fig. 6ii). Once again, there were not dramatic changes in the vaccine A profiles at 37 °C or 55 °C. This same detergent extraction changes the particle distribution profile of vaccine B with two significant peaks (122 nm and 160 nm) as well as smaller shoulders at 190 nm and 280 nm respectively (Fig. 6iv). Heating vaccine B in the presence of zwittergent decreases particle size, with the sample held at 55 °C closely resembling the distribution observed for untreated vaccine A (Fig. 6iv, Table 3). This suggests that the submicron-level aggregates have been dissociated by this treatment.

If these aggregates are responsible for the lack of antigenic recognition of HA that we observe, then their dispersal should allow for a recovery of this detection. Because the dispersal of aggregates requires thermal treatment at a level expected to cause conformational changes in HA trimers we used lectin to capture and a non-conformationally sensitive antibody (RP03) as a detection agent. When the vaccine is initially treated with zwittergent there is a marginal increase in the detection of HA, this becomes more pronounced as the temperature is raised. As suggested by NTA when we treat vaccine B with 1% zwittergent 3–14 and hold it at 55 °C there is a large shift in the available HA with significant amounts now detectable (Fig. 7). This finding demonstrates both that the aggregation is linked to the inability to bind HA in this vaccine and that dissociation of these aggregates recovers this binding.

4. Discussion

In this paper we investigated a novel ELISA technique to quantify HA in inactivated influenza vaccines. Since 1978 inactivated influenza vaccines have been formulated and tested for HA content by SRID [34,35]. There are significant strengths to the SRID that have been well described elsewhere, however to summarize, these include detection of antigenicity, subtype-specificity and a low level of technological sophistication [4,36]. As with any technique there are also disadvantages, some of which include a constant requirement for a timely supply of updated reagents, a lack of sensitivity at low levels of antigen as well the SRID is a relatively low-throughput and labour-intensive assay. While numerous alternative and complimentary methods have been suggested in response to these shortcomings these various assay types remain incompletely characterized. In this report we demonstrate that antibody-based evaluation of influenza potency by a capture from a liquid environment can be impacted significantly by protein agglomeration.

Initially, we tested standards and vaccines using a lectin-based platform to bind both HA and NA from inactivated vaccines allowing them to be measured by specific HA or NA antibodies. The technique proved robust at detecting and quantifying HA in monovalent vaccines. The chief advantage of using lectin as a capture reagent is that it provides a universal binding platform and when combined with group specific antibodies can be used to probe and quantify any monovalent influenza vaccine. While the results demonstrated that widely available commercial reagents can be used to construct a straightforward potency assay based on structural conformation they also pointed to some potential difficulties. One manufacturer's product was largely unquantifiable due to the presence of significant aggregates of submicron size. Although these aggregates rendered the HA unquantifiable by ELISA, the HA was both antigenic and immunogenic in terms of SRID analysis and mouse immunogenicity studies. While the differences in HI titre generated by the different vaccines did not reach a minimum threshold of significance ($P < 0.05$) at identical doses we can observe significant differences when we compare 10-fold differences in dose level reinforcing the fact that we do have the power in this study to see large differences in vaccine immunogenicity. The SRID test also indicates that these vaccines have similar potency, so the question became what is interfering with the ability of this ELISA to evaluate the potency of this HA? Our immunoprecipitation/RP-HPLC results revealed that all antibodies tested could capture the HA from vaccine A, but none could precipitate any significant amount of HA from vaccine B. This is despite the fact these antibodies clearly recognize the HA in this vaccine when it is separated on an SDS-PAGE gel or analyzed through SRID. When we treated vaccine B in a manner that largely dissociated the aggregates, we could restore antigenic recognition in the ELISA format. Unfortunately, in our hands this dissociation required the use of conditions that will alter the confirmation of the HA trimers contained in the vaccine rendering this treatment inappropriate for potency determination.

A protein aggregate is defined as any self-associated complex of monomers where the monomer is the "naturally occurring and/or functional subunit" and include agglomerations ranging from a few oligomers to a few mm in size [37]. Aggregation of therapeutic

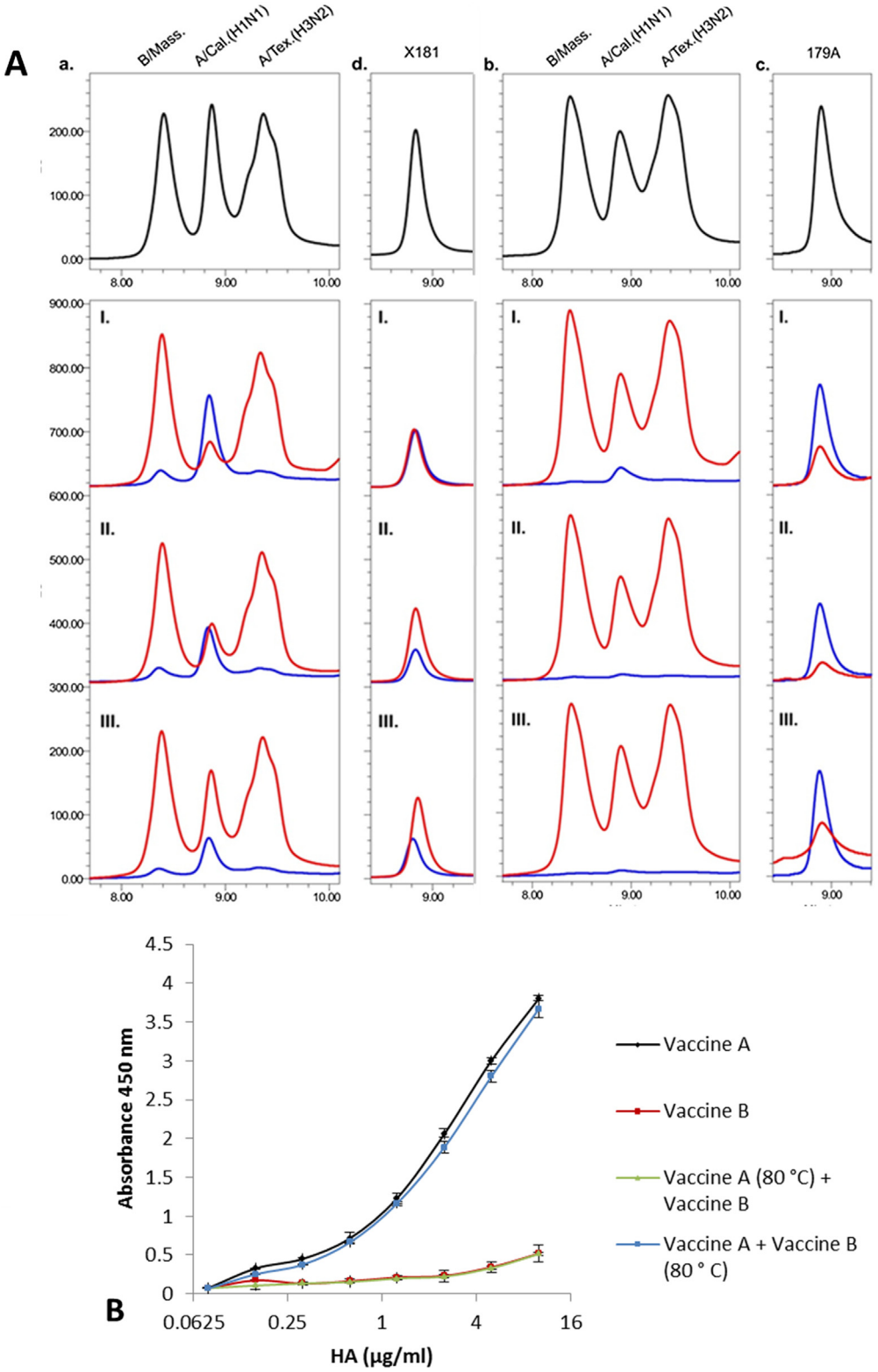

Fig. 5. Immunoprecipitaton of HA measured by HPLC. (A) Trivalent vaccine A (a), B (b) NIBSC reference standards X179A (c) and X181 (d) were each incubated with MMAb C179 (i), RPab RP-03 (ii) Sheep anti-sera 16/144 (iii) respectively before immunocomplexes were precipitated with Protein A/G and the unbound fraction separated. Each sample was subsequently examined by HPLC as described in [24] and the strains of HA (B/Mass, A/Cal, and A/Texas) are separated and identified. The trace for the immunoprecipitated HA is presented in blue, the uncaptured fraction in red. Displayed in black are the trace for the maximal HA signal for each sample as provided by HPLC analysis of an unprecipitated sample. **(B)** Vaccines were treated at 80 °C for an hour to provide a vacine diluent devoid of detectable HA activity. Inactivated vaccine A or an identical volume of PBS were mixed with vaccine B to make TV A (80 °C) + TV B or TV B respectively. Inactivated vaccine B or an identical volume of PBS were mixed with vaccine A to make TV A + TVB (80 °C) or TV A respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

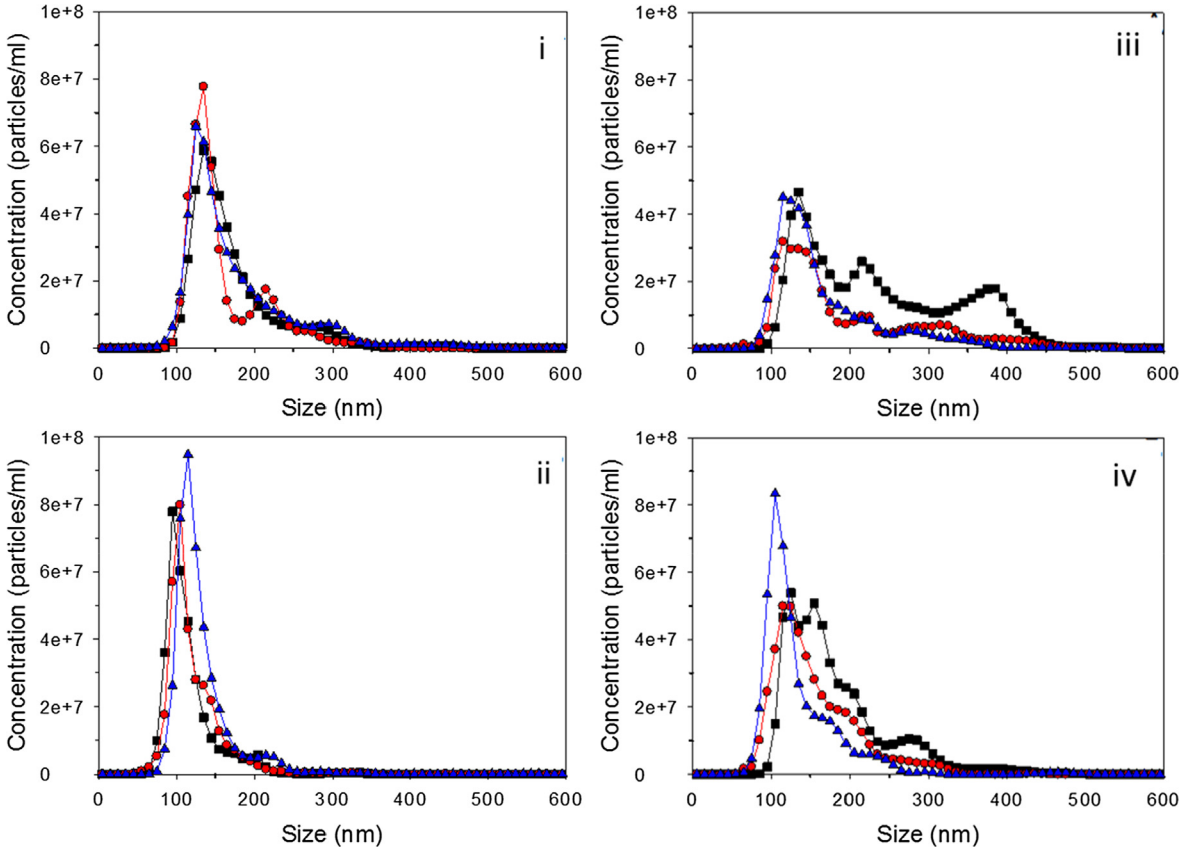

Fig. 6. Nanoparticle Tracking Analysis. Number weighted size distribution analysis of trivalent A (i & ii) and B (iii & iv) influenza vaccine samples with single nanoparticle tracking analysis. Incubation at 20 °C (black squares), 37 °C (red circles) and 55 °C (blue triangles) for 60 min in the absence (I & iii) or presence (ii & iv) of 1% w/v Zwittergent 3–14 Plots are representative of 3 separate experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Comparison of Treatments and particle distribution. Summary of NTA data collected in triplicate on indicated vaccines with listed treatment.

Sample	Vaccine B 20 °C	Vaccine B 20 °C + Zwittergent	Vaccine B 37 °C	Vaccine B 37 °C + Zwittergent	Vaccine B 55 °C	Vaccine B 55 °C + Zwittergent
Mean	227.6 ± 6.7	171.5 ± 4.1	195.6 ± 9.4	153.4 ± 3.7	168 ± 8.0	128.3 ± 4.9
Mode	131.2 ± 6.2	122.3 ± 4	124.3 ± 8.3	132.7 ± 21	123 ± 7.0	112.2 ± 7.8
D10	113.8 ± 4.7	107.9 ± 2.3	98.6 ± 1.5	96.8 ± 5	94.8 ± 8.5	83.7 ± 1.4
D50	202.2 ± 5.4	153.9 ± 4.4	149.3 ± 2.2	135.3 ± 6.1	128.8 ± 12.8	109.7 ± 4.4
D90	367.2 ± 6.9	255.6 ± 9.2	345.3 ± 12.3	217 ± 2.7	283 ± 28	182.2 ± 6.7
Sample	Vaccine A 20 °C	Vaccine A 20 °C + Zwittergent	Vaccine A 37 °C	Vaccine A 37 °C + Zwittergent	Vaccine A 55 °C	Vaccine A 55 °C + Zwittergent
Mean	162.4 ± 3.7	105.9 ± 5.1	158.1 ± 1.6	122.3 ± 9.3	165.3 ± 5.1	123 ± 7.9
Mode	132.5 ± 5.8	97.6 ± 3.2	131.3 ± 2.8	105.3 ± 6.1	128.1 ± 4.3	108.3 ± 5.2
D10	107.8 ± 5.4	71.8 ± 4.9	107.9 ± 3.0	84.6 ± 5.7	102.3 ± 3.5	85 ± 5.6
D50	138.1 ± 5.2	92.4 ± 3.7	134 ± 2.7	106.3 ± 12	141.4 ± 4.5	107.8 ± 5.6
D90	239.2 ± 13.5	137.3 ± 14	224.6 ± 8.2	163.3 ± 9.2	253.1 ± 15.2	161.4 ± 12.8

biologic products is complex and the effect on immunogenicity highly dependent on the nature of the aggregation as well as the product in question [38]. While the clinical effect of aggregation can range from a negative or lowered immune response due to loss of efficacy and/or misdirection of the intended immune response (such as changing the balance of Th1 and Th2 cytokines produced) [39–42] it can also result in an amplified or improved immune response to the aggregated product and the induction of aggregation can be used as an adjuvant in vaccine development [43–45]. Some studies have demonstrated that induction of neutralizing antibodies can be correlated with aggregation levels and that too few aggregates results in the induction of immune tolerance in some therapeutic products [46,47]. The mechanisms by which these changed responses are induced are equally varied and include shifting the immune response to less relevant targets, greater cross-linking of immune receptors, increased triggering by pathogen-associated molecular patterns (PAMPs) or other danger signals and enhancing antigen uptake and presentation by APCs [48,49]. Furthermore, the immune pathway amplified and the robustness of the response is going to be highly dependent on the fundamental nature of the aggregate itself. Factors such as size (<100 nm (nanometer or soluble), 0.1–1 μm (submicron or soluble), 1–100 μm (subvisible) or >100um (visible), conformation (native, unfolded, misfolded, etc.), reversibility/dissociation, chemical modification and morphology in addition to the protein itself shape or guide this reaction [38].

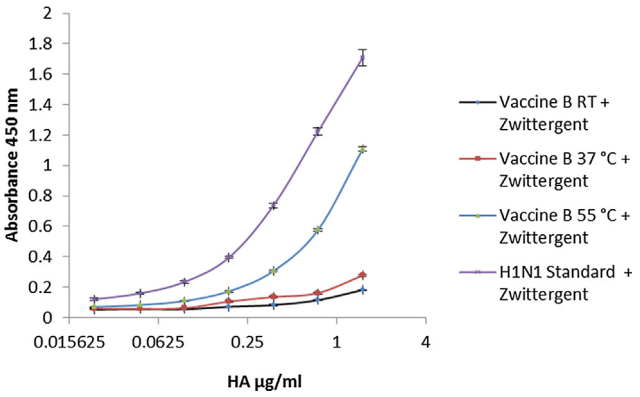

Fig. 7. ELISA analysis of dissociation of aggregates. Samples of influenza trivalent vaccine B were treated at 20 °C, 37 °C or 55 °C for 60 min in the presence of Zwittergent 3–14. These samples were then diluted 1:10 and analyzed by ELISA technique on a plate coated with 10 µg/ml *Crotalaria juncea* lectin. A/California/7/2009 was detected by Rabbit polyclonal antibody RP01.

The first direct evidence of aggregation in vaccine B was demonstrated on SDS-PAGE gels where we observed a number of bands of greater molecular weight than expected under both reducing and non-reducing conditions. Western blots from these gels verified that the H1 HA from vaccine B is contained in some of the higher bands which correspond in size to HA monomers dimers, trimers and even larger oligomers. The same is not true in vaccine A in which the HA antigen (H1/California/2009) behaves as expected when run on reducing and non-reducing gels, or in other analysis of similar antigens [22,50]. The fact that HA monomers remain strongly associated under reducing conditions implies the presence of additional covalent interactions between the two HA1 and HA2 monomers yet we have found no evidence for differential protein modifications and the protein sequence were as expected. This favours the hypothesis that these aggregates are instead being formed through hydrophobic interactions. A change in the interaction between HA1 and HA2 could alter the native trimeric structure enough to provide a nucleation site for agglomeration with additional HA trimers. The generation of aggregates of HA trimers may be providing some protection against detergents, thermal treatment and reducing agents that we observe by SDS-PAGE gels and western blots. By nanoparticle tracking analysis we observed that vaccine B was comprised of fewer but larger particles with sizes ranging up to 400 nm or submicron size. The particles we detect begin to be broken up through relatively minor treatment (37 °C for an hour) although nearly complete dissolution required elevated heat in the presence of detergent indicative that these are aggregates of smaller particles. It is unclear if these represent distinct populations of aggregates or are simply a spectrum of aggregated proteins. Nor can we tell the relative contribution of HA trimers or other influenza proteins to any individual particle or population nor the relative contributions of H3 and B HA trimers to these aggregates. What we can state is that the dissolution of these submicron sized aggregates correlates with the ability to bind HA by immunocapture techniques but that the methods we use to cause disaggregation are harsh enough that we render the vaccine unusable for pre-treatment potency determination.

As established by both the United States Pharmocopeia (USP) and the European Pharmocopeia (EP), biotherapeutics that are injected need to be free of visible particles (>50 µm) and strict limits are placed upon the quantity of subvisible particles that are accepted. In practical terms however, this statutory limit applies to particles>10 µm. Due to the difficulty in quantifying aggregates across submicron levels as well as the inherent difficulty in establishing a specific release limit that applies to all products aggregates in this range are qualitatively assessed rather than quantified [51–53]. This study provides evidence that different influenza HA potency assays will provide very different potency estimates when confronted with HA in a preparation in which there is significant aggregation. In our hands SRID is capable of distinguishing the conformation of this aggregated HA be it native trimers or misfolded/denatured and will quantify the HA at least in the presence of sub-micron level aggregates [54]. Potency assays which evaluate vaccines by taking a portion of the vaccine and using that as a surrogate for the antigenicity of the whole may face difficulties when dealing with a wide variety of manufacturing techniques and protocols. Conversely those assays which measure HA through analysis a larger portion of the vaccine antigenic components may better estimate the total immunogenic HA contained in wide variety of products even if the value they assign to a specific fraction over- or under- estimates its specific contribution [12,55,56].

In the SRID test, 1% zwittergent, is added in order to disrupt virions or virus-like particles and extract the HA allowing trimers or small oligomers to diffuse through the gel and precipitate. Our results demonstrate that treatment with zwittergent is not sufficient, on its own, to extract the HA from these aggregates suggesting that something else in SRID method enables detection. One possibility is that these aggregates themselves are diffusing through the agarose gel but with reduced mobility relative to the liquid environment and once in this restricted environment are capable of retaining an interaction with the antibodies contained therein. However, we can't rule out the possibility that these submicron-sized aggregates themselves are being further dissociated by their diffusion through the agarose matrix. Conversely, the lack of detection of aggregated HA by ELISA or immunoprecipitation suggests that either the binding affinity is insufficient to couple the antibody to the larger mass or the stearic hindrance is such that the antigen and antibodies do not maintain their interaction in a liquid environment. It is possible that these assay differences can be used to better characterize inactivated influenza vaccines. For example, if a vaccine were to contain a mixture of HA in oligomers and aggregated trimers the difference between the total antigenic/protected HA and the free HA trimers available for binding/capture may provide a reasonable estimate of the quantity of HA contained in these aggregates.

In vaccine B the aggregates we find range in size to approximately 400 nm and are quantifiable by SRID but not quantifiable when we use an immunocapture type assay. Formulating or measuring a vaccine with either approach alone provides an incomplete picture of the immunogenic HA contained in the vaccine. Recently Wen *et al.* published a study detailing how both proteolysis and SRID were able to predict some *in vivo* immunogenic changes while failing to co-relate with others when different physical stresses placed on an influenza vaccine [56]. Much like we find in this study, different methods of characterizing HA can produce *in vitro* results which don't always align with the *in vivo* outcome. Therefore, seeking to replace the SRID with any other single *in vitro* approach is not likely to improve the predictability of the immunogenicity of inactivated influenza vaccines. A better approach, and one which may provide increased consistency in these products, would be to complement SRID analysis with a well characterized orthogonal method.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.07.050.

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