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A novel assay for influenza virus quantification using surface plasmon resonance

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ABSTRACT

Quantification of hemagglutinin (HA) by single-radial immuno diffusion (SRID) is the predominant method to ensure product potency in seasonal influenza vaccines. Here a new method for quantification of influenza virus using biosensor technology is presented. The method employs quantification of virus via an inhibition assay format using HA proteins for H1N1, H3N2 and B immobilized on a sensor chip. Initial results showed the assay to have higher sensitivity (detection range 0.5–10 μg/ml), higher precision and significantly lower analysis and hands on time compared with SRID.

1. Introduction

Vaccination against influenza virus is the primary strategy to reduce the mortality associated with seasonal influenza. The antigen content of the influenza vaccines is predominantly determined during development and manufacturing by quantification of hemagglutinin (HA) using immunochemical methods. The most commonly used and important method for this purpose is single-radial immuno diffusion (SRID). SRID has been used to determine the potency of all human inactivated influenza virus vaccines licensed by the Food and Drug Administration (FDA) since 1978 and is also the method recommended by the World Health Organization (WHO) and the European Agency for the Evaluation of Medicinal Products (EMEA) [1–3]. However, in addition to being very labor-intensive, this method has several other disadvantages including low accuracy, precision and sensitivity [4].

So far influenza vaccine has been mainly produced using fertilized eggs. However, the fear of a new pandemic of influenza virus has initiated the development of a more efficient production of vaccines using cell culturing. This process development has increased the demand for new sensitive and simple analytical tools.

Many other methods for influenza virus quantification and detection have been reported: enzyme linked immunosorbent assays (ELISA) [5–7], neuraminidase (NA) activity assays [8,9], HPLC methods [10–12], hemagglutination assays [11,13] and quantitative PCR (qPCR) [14–16]. Some of these methods show increased sensitivity compared to SRID. However, with the exception of multiplex qPCR, these methods do not reduce the hands on time nor can they simultaneously quantify HA from three different subtypes in a single experiment. qPCR is more useful for detection and subtyping of influenza virus than for quantification, due to the mandatory conversion step from RNA to DNA. Williams et al. [17] presented a liquid chromatography–tandem mass spectrometry technique with isotope dilution for absolute quantification of viral proteins. The advantages of this method are the possibility to analyze a trivalent vaccine in one assay as well as the limited need for reference antigens and sera. However, since independent reference standards for determining HA concentrations were not available, a direct comparison with SRID measurements was not possible. In addition, the method also required sample preparation prior to analysis [17].

Previous reports on the use of surface plasmon resonance (SPR) for detection and quantification of influenza virus were all based on a direct binding approach [18–25]. These studies either measured the kinetics of HA binding to its sialic receptor [20,22], or the affinity of antibodies to HA and NA [18,19,21] or described the selection of RNA aptamers against HA [23]. One report described a method for quantifying influenza virus in process samples and vaccines [25]. This method which used sialic acid based compounds as weak affinity ligands generic to several virus strains had an analytic range of 10–100 μg/ml. It used a continuous weak affinity biosensing approach which obviated the need for regeneration and reduced the analysis time [25]. A miniaturized biosensor has also been tested for avian influenza virus detection with a limit of detection of 5 μg/ml for the specific antigen [26]. Although these SPR

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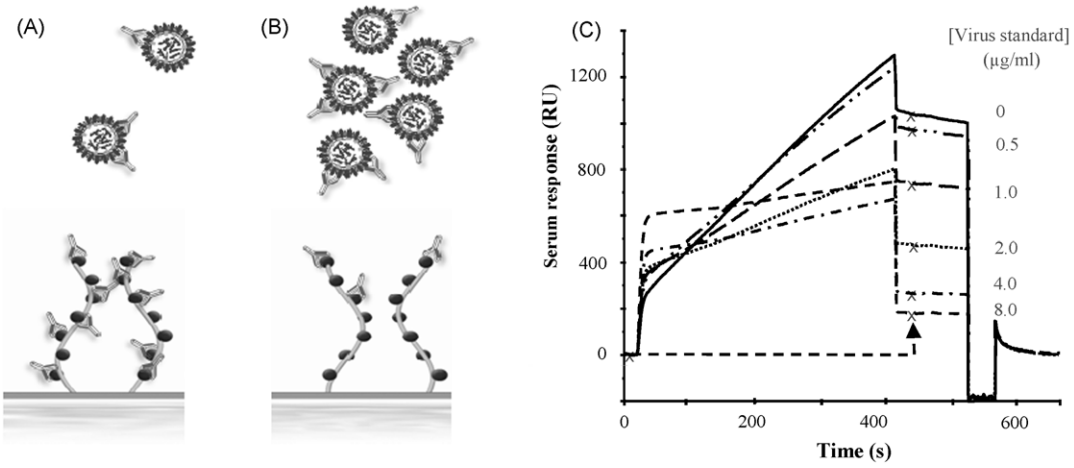

Fig. 1. Inhibition assay principle (A and B). HA is first immobilized on the dextran matrix (black filled circles). Virus is then mixed with a fixed concentration of serum and injected over the surface. Free antibodies (not bound to virus at equilibrium) bind to the surface HA, giving a response. Low concentration of virus in the sample (A) gives high antibody binding, while high virus concentration (B) results in low binding level. (C) Overlay plot showing sensorgrams of injected serum mixed with a concentration series of virus standard. Report points (marked as X) are taken before and after injection and response levels are measured between those (arrow). After each injection the surface is regenerated in preparation for a new injection.

based methods decreased the analysis hands on time, they were not more sensitive than SRID and did not have the capacity to analyze three virus strains simultaneously.

Here a new method using surface plasmon resonance for accurate quantification of influenza virus in an inhibition format is presented. Recombinant HA proteins [27] (A/H1N1, A/H3N2 and B respectively) were immobilized on the dextran matrix of a sensor chip, and free antibodies in a mixed solution of virus antigen and serum, specific for the three influenza types, were allowed to bind to the HA protein on the surface, giving rise to a change in the response (Fig. 1). The higher the concentration of virus in the sample, the lower the residual binding response. The recombinant HA proteins were immobilized in three different flow cells on the same chip, making it possible to analyze trivalent vaccines in the same experiment. The biosensor assay was shown to be robust, to have higher recovery, precision and sensitivity, and a shorter analysis time than SRID. The calibration curve range was 0.5–10 μg/ml and the limit of detection was estimated as <0.5 μg/ml for all three subtypes A/H3N2, A/H1N1 and B. As the reference antigens and sera used in this method are the same as the ones used today for vaccine potency assays, this new method has the power to complement and/or replace SRID as an influenza vaccine quantification assay in the future.

2. Materials and methods

2.1. Reagents

Reference sera (sheep) and virus antigens were acquired from NIBSC, UK, except for the B/Brisbane/3/2007 serum and antigen that were received from Solvay Pharmaceuticals, The Netherlands, and the PR/8/34 serum (chicken) and virus that were acquired from Charles River Laboratories, USA. Recombinant full length HA proteins (A/H1N1/New Caledonia/20/99, A/H3N2/Wyoming/3/2003 and B/Jilin/20/2003) were acquired respectively from ProspecBio, Israel, from Genway, The Netherlands and from Protein Sciences, USA. The process samples analyzed in Table 4 were from GE Healthcare, Sweden, except for the B/Brisbane/3/2007 (MBV and TBV) samples and the A/H1N1/Solomon Islands/3/200 TBV sample which were provided by Solvay Pharmaceuticals, The Netherlands. Trivalent vaccines were acquired from three commercial manufacturers. Surfactant P20 (polyoxyethylenesorbitan), HBS-EP+ buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% Surfactant P20), Amine coupling kit, Cy^3-Dye, agarose and λ-DNA were from GE Healthcare. Bovine serum albumin (BSA) was from Pierce, DMEM from Invitrogen and Sucrose, NaCl and Zwittergent from Merck.

2.2. Biosensor system

Biacore™ T100 system and Sensor Chip CM5 (GE Healthcare) were used for all analyses. Interaction studies are based on surface plasmon resonance detection at gold surfaces, in combination with a microfluidic system for reagent handling. The interaction between biomolecules is monitored in real time using label-free assay formats. One of the interacting molecules is immobilized to a semi-fluidic dextran matrix on a sensor surface. The other molecule is then injected in solution over the surface. As molecules in the injected sample bind to the interacting partner immobilized on the sensor surface, a change in SPR response is detected. The change in response level is proportional to the change in mass at the surface.

2.3. Biosensor quantification assay

The method was set up as an inhibition assay (Fig. 1) to avoid diffusion effects of the large virus molecules [28]. Samples were analyzed without pretreatment. HBS-EP+ was used as analysis buffer. Recombinant HA proteins were immobilized to 7000–10,000 response units (RU) using standard amine coupling protocol [29], with injection of 50–80 μl rHA (10 μg/ml) in 10 mM maleate buffer, 0.05% Surfactant P20, pH 6.5 (15 min for HA/H1N1 and 7 min for HA/H3N2) or in 10 mM phosphate buffer, 0.05% Surfactant P20, pH 7.0 (20 min for HA/B). Anti-influenza sera were titrated to give responses of 700–1400 RU for a 400 s injection on respective surfaces. Mixtures of virus reference antigen, or a virus sample with unknown concentration, and a fixed dilution of anti-influenza serum were injected during 400 s, followed by regeneration of the surface using either 50 mM HCl, 0.05% Surfactant P20 (30 s).

Free antibodies in the injected sample bind to HA on the surface giving rise to a response (Fig. 1). Five to 10 startup cycles with serum only and regeneration were run first in each analysis to stabilize the surface. Calibration curves with serum and reference antigens were analyzed first, in the middle and at the end of each assay. To adjust for shift in the calibration curve during analysis the equation factors from the four parametric fit regressions of the three calibration curves were used for normalization with a proto-

Table 1
Calculated concentrations and coefficient of variation for analysis of B/Jiangsu/10/2005 (Fig. 3).

Ref[a] [HA] (μg/ml)	Biosensor [HA] mean (μg/ml)	CV (%) $n = 5$
1.0	1.0	3.7
2.0	2.0	5.0
4.0	3.9	3.3
6.0	6.1	2.0[b]
8.0	8.1	2.5

[a] By manufacturer.
[b] $n = 28$.

type functionality in the biosensor software. Thereby each sample received a unique set of calibration curve factors for calculation of the sample concentration.

Standards and samples were prepared in a microtiter plate format with sample volumes of 40–80 μl, and analysis was typically run unattended over night (ca 15 h for 96 samples/standards).

The additives used for robustness testing in Table 3 were λ-DNA, BSA, DMEM, sucrose and NaCl.

In the trivalent assay HA A/H1N1, A/H3N2 and B were immobilized in three different flow cells on the same chip (Fig. 7). The three specific reference antigens and sera (A/H1N1/Solomon Islands, A/H3N2/Wisconsin, B/Malaysia) were first mixed to form a trivalent standard curve antigen and a trivalent serum. The samples were allowed to pass over the three surfaces, thus analyzing all three strains simultaneously in one experiment.

The sera used to investigate subtype specificity were diluted in proportion to titers for SRID, provided by the manufacturer, to take into account differences in serum affinities.

2.4. SRID

Sera were labeled with Cy^3-Dye. Unlabeled sera and Cy^3-labeled sera (less than 0.5% of the total serum amount; was found not to interfere with the precipitation) were mixed with melted agarose (1%) and cast in a mould creating holes in the gel. Samples and reference antigens were treated with Zwittergent (1%, 30 min at ~22 °C) prior to being added to the holes in the gel. The gels were incubated for 15–18 h at ~22 °C, and subsequently dried and scanned at 450 nm in Typhoon. Evaluation was performed by comparing ring areas of samples with reference antigens using ImageQuant TL software. Instruments and software were from GE Healthcare.

3. Results

3.1. Assay calibration and limit of detection

Fig. 2 shows typical calibration curves for the three assays: B, H3N2 and H1N1 respectively. The dynamic range of the calibration curves was optimized to 0.5–10 μg/ml giving an recovery of 98–102%. The limit of detection was estimated as <0.5 μg/ml and the limit of quantification ~1 μg/ml for all three subtypes, based on dilution series of the reference antigens down to close to 0 μg/ml. Fig. 3 shows the relative response of repeated injections of B/Jiangsu/10/2005 serum and antigen at different concentrations (0.5–16 μg/ml) on the recombinant HA B surface. Calculated concentrations are presented in Table 1.

A decrease in response with time was noted, which was greatest for the first 10–20 cycles and then stabilized with increased cycle number. A similar effect was detected for all tested reference antigens on their respective surfaces and it was unaffected by changing regeneration conditions or by pretreating the samples with detergents (data not shown). To increase the accuracy of analysis three calibration curves were run and the sample concentrations were determined from interpolated calibration curves as described in Section 2.

3.2. Subtype specificity

To investigate the specificity of interaction of serum directed to different strains with the recombinant HA proteins on the sur-

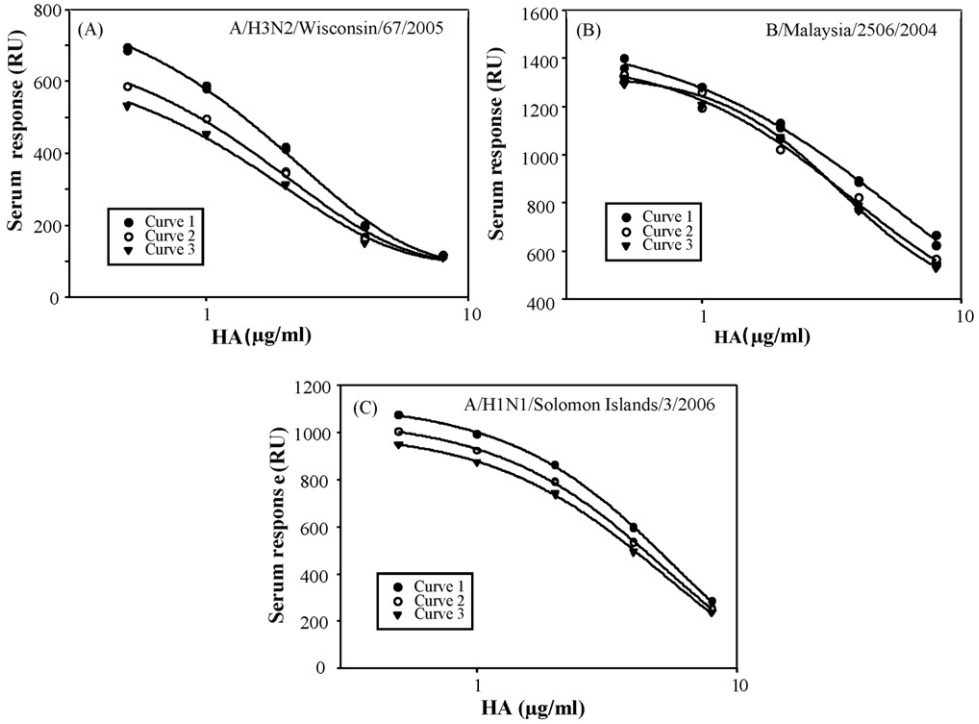

Fig. 2. Calibration curves for A/H3N2 (A), B (B) and A/H1N1 (C) with concentration range 0.5–8 μg/ml of each reference antigen reagent in corresponding serum. Calibration curves were run first, in the middle and last in assays with ~40 unknown samples.

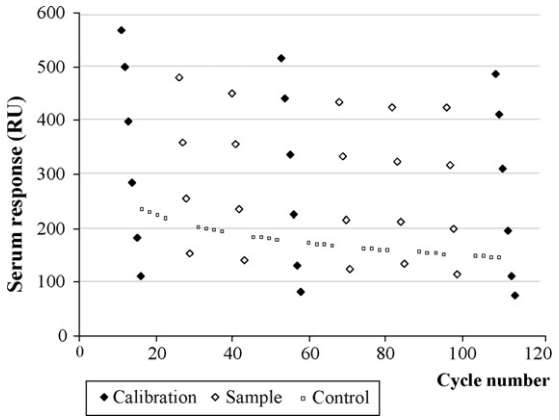

Fig. 3. Serum response levels from a quantification of B/Jiangsu/10/2005. Reference antigen was diluted and mixed with corresponding serum to concentrations between 0.5 and 16 μg/ml. Calibration points (filled diamond), samples (open diamond) and a control sample (6 μg/ml, open square) were analyzed repeatedly on a surface immobilized with recombinant HA B. Calculated concentrations are presented in Table 1.

face, antisera of different strains were analyzed on all three surfaces (Fig. 4). Sera were tested with and without reference antigen at 2 μg/ml, to check that the response was specific for the respective virus and that the virus itself did not cause unspecific background effects. The results showed that the three recombinant HA proteins immobilized on the surface were specific for the three sera subtypes A/H1N1, A/H3N2 and B respectively, but were generic for strains within these subtypes of the virus. Only one serum of all those tested bound to all surfaces (chicken A/H1N1/PR/8/34 serum, data not shown). The reason for this is at present unknown. It is possible that a chicken derived component resulted in unspecific response, as all other sera were from sheep.

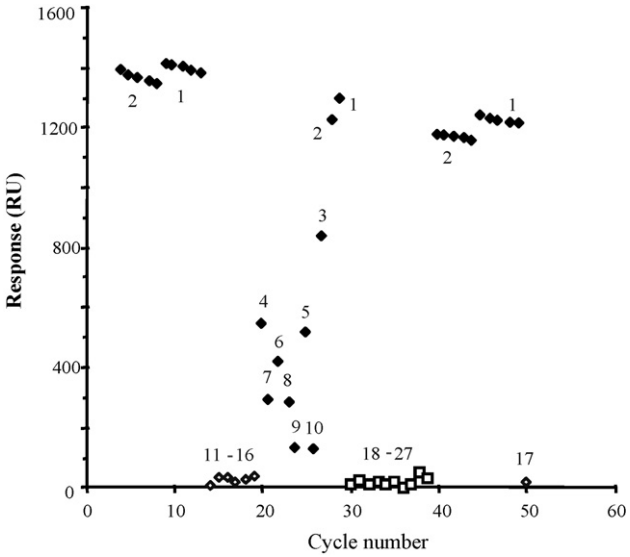

Fig. 5. Responses from 27 different sera, specified in Table 2, on sensor chips with recombinant HA/H3N2 immobilized. Binding of A/H3N2 serum strains is marked by filled diamonds, A/H1N1 serum strains by open diamond and B strains by open squares. The surfaces were regenerated with 50 mM HCl, 0.05% P20. Sera 1 and 2 were analyzed in replicates to monitor response drift.

To expand the specificity study the binding of 27 different sera to a surface immobilized with HA/H3N2 was studied (Fig. 5, Table 2). Results showed that only sera specific to the H3N2 subtype gave significant binding to the surface. The anti-B- and anti-H1N1 sera showed only low binding levels. Correspondingly, experiments using regeneration with 50 mM HCl, 0.05% P20 were performed with 27 sera also on surfaces with HA/B and HA/H1N1 immobilized. Results showed significant bind-

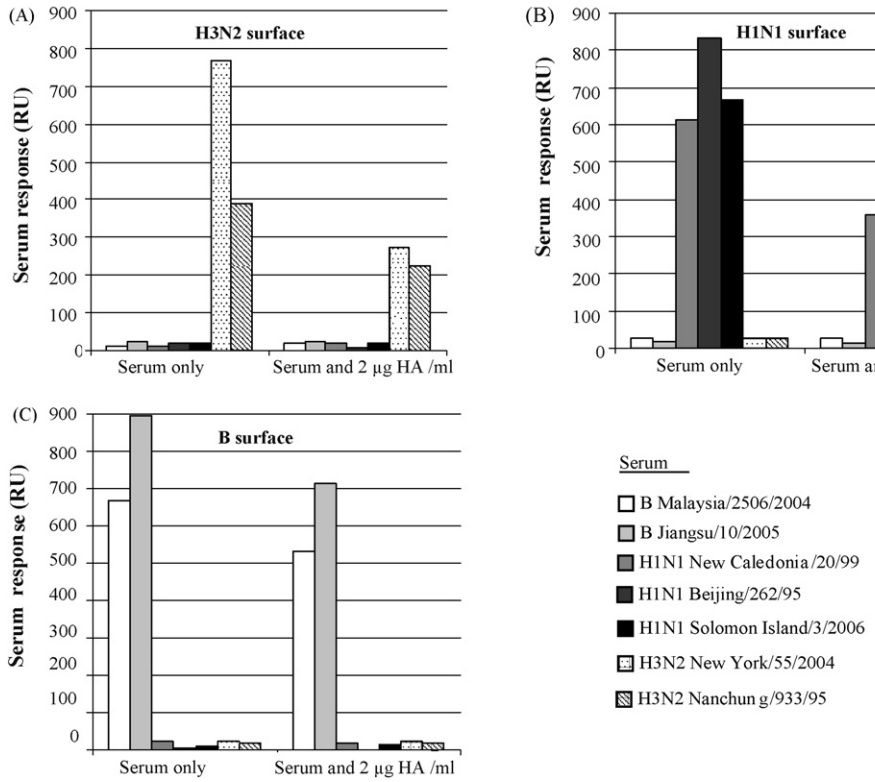

Fig. 4. Influenza subtype specificity. Binding levels of different strains of sera were analyzed on three surfaces, A/H3N2 (A), A/H1N1 (B) and B (C), in the same experiment. The samples contained either serum, or serum mixed with 2 μg/ml of respective reference antigen.

Table 2
Serum name and identification number.

H3N2		H1N1		B	
ID	Serum	ID	Serum	ID	Serum
0	Nanchung	11	Chile	18	Ann Arbor
1	Wyoming	12	New Jersey	19	Beijing
2	New York	13	New Caledonia	20	Guangdong
3	Sichuan	14	Solomon Islands	21	Harbin
4	Beijing	15	Taiwan	22	Johannesburg
5	Shanghai	16	Beijing	23	Panama
6	Guangdong	17	Brazil (goat)	24	Shangdong
7	England			25	Yamanashi
8	Leningrad			26	Jiangsu
9	Mississippi			27	Malaysia
10	Shangdong				

ing of only anti-B- and anti-H1N1 sera respectively (data not shown).

3.3. Strain specificity

Initial analyses were performed to explore strain specificity. Calibration curves with the four possible combinations of standard and serum for the two randomly chosen H3N2 strains Wyoming and New York, were run on a sensor chip with recombinant HA A/H3N2/Wyoming immobilized (Fig. 6A). The lowest concentrations had response levels in the range 1700–2000 RU. In order to facilitate comparison, responses were normalized by dividing each response with that of the lowest concentration (in %), for respective curve. The curves were run in order: N.Y./N.Y., N.Y./W, W/W and W/N.Y. Four replicates of the combination W/W were run on another chip with similar immobilization level, providing reference data of experimental variation (Fig. 6B). Results showed inhibition for all combinations with W/W allowing for the highest sensitivity. Results also show that in the case of these two strains, if, e.g. New York standard and serum were to be used for quantification of Wyoming samples; too high calculated concentrations would be obtained.

3.4. Robustness

During vaccine purification, samples from various steps in the process will have a different purity profile and may include compounds that possibly interfere with the concentration analysis. To investigate the influence of additives on the analysis, BSA, λ-DNA, cell culture medium, sucrose and salt were added to reference antigens prior to analysis. The results are shown in Table 3. BSA, λ-DNA and cell culture medium did not interfere with the analysis. Salt and sucrose decreased responses, increasing the apparent concentration markedly; however the effect of these additives could be avoided by the dilution process. A concentration of <1% sucrose and <0.2 M sodium chloride was found to be desirable for robust quantification.

Table 3
Influence of additives on recovery. Reference antigen B/Jiangsu/10/2005 (5 μg/ml) was mixed with additives and the samples were analyzed on a B surface. Results were compared to control samples without additive in HBS-EP+ buffer.

Additive	Compared with expected (%)	CV (%) $n = 3$
Control (antigen in HBS-EP+)	100	4.1
BSA 0.13 mg/ml	91	4.7
BSA 0.25 mg/ml	91	9.8
BSA 0.5 mg/ml	95	na
BSA 1 mg/ml	97	na
λ-DNA 0.125 μg/ml	94	6.6
λ-DNA 0.25 μg/ml	90	4.8
λ-DNA 0.5 μg/ml	96	0.4
λ-DNA 1 μg/ml	98	3.4
λ-DNA 10 μg/ml	96	1.8
Cell culture medium 0.01%[a]	104	0.7
Cell culture medium 0.1%	106	0.9
Cell culture medium 10%	99	na
Sucrose 0.25%	98	3.3
Sucrose 0.5%	104	0.8
Sucrose 1%	116	3.0
Sucrose 5%	130	2.5
NaCl 0.15 M	100	4.1
NaCl 0.3 M	150	0.4
NaCl 0.5 M	166	1.5
NaCl 1 M	178	0.4

[a] Dulbeccos modified Eagles medium.

Precision was studied running replicates of a control sample containing purified A/H1N1/Solomon Island (55 μg/ml). Within one analysis (one chip/gel) the biosensor method resulted in an intra CV of 2% (5 replicates) compared with 9% (12 replicates) using SRID. Between different analyses made different days by two operators the biosensor method resulted in an inter CV of 5% (3 analyses) compared with 18% (5 analyses) for SRID.

3.5. Analysis of process samples

To test the performance of the biosensor method, samples from different steps in an influenza vaccine purification process were analyzed, from harvest of cell culture supernatants to filtered end

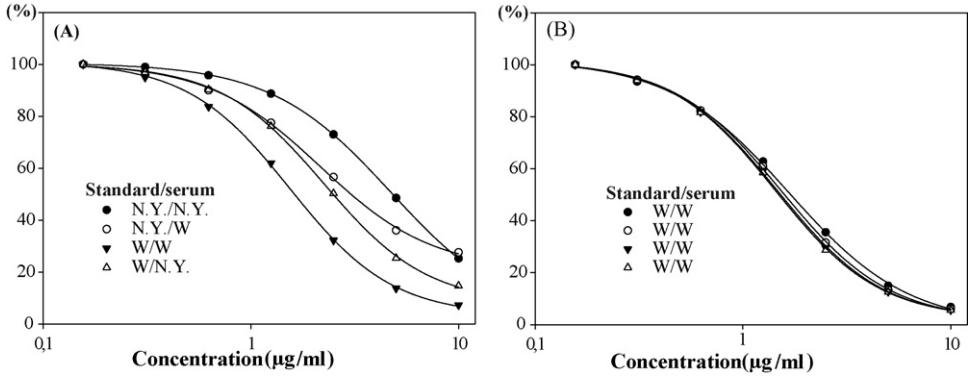

Fig. 6. Example showing strain specificity on sensor chip with recombinant HA A/H3N2/Wyoming immobilized. Calibration curves were run with the four possible combinations of standard and serum for the two H3N2 strains Wyoming and New York. Responses were normalized for comparison, by dividing each response with that of the lowest concentration in %, for respective curve. Curves are named after respective standard/serum used, e.g. W/N.Y. means that standard Wyoming and serum New York was used for that curve (A). Four replicates of the combination W/W were run on another chip with similar immobilization level, providing reference data of experimental variation (B).

Table 4
Quantification of process samples; results from biosensor and SRID.

Strain		Sample type	Biacore [HA] (μg/ml)	CV (%) $n=2$	SRID [HA] (μg/ml)	CV (%) $n=2$
B	Brisbane/3/2007	TBV[a]	31	0.4	33	6.5
		TBV[a]	43	0.6	36	5.2
		TBV[a]	43	1.7	30	7.6
		MBV[a]	260	0	244	16
A/H1N1	Solomon Islands/3/2006	Harvest	8.3	10	8.0	7
		UFD	78	3.8	76	5.4
		TBV[a]	50	3.5	40	5
	New Caledonia/20/99	Start 1	47	2.0	58	11
		Start 2	18	1.3	23	na
		Eluate 2	63	1.9	61	6
		Start 3	20	1.8	22	31
		Eluate 3	47	1.0	45	17
	PR/8/34	Harvest	7.4	1.0	<LOD	na
		UFD	20	9.3	19	21
A/H3N2	Wisconsin/67/2005	Harvest	1.3	4.3	<LOD	na
		NFF	1.0	2.1	<LOD	na
		DF	4.3	1.4	<LOD	na
		TBV	28	0.2	27	3.0
		TBV	38	0.3	35	8.2

TBV, trivalent bulk vaccine; MBV, monovalent bulk vaccine; harvest, supernatant from infected MDCK cell culture; UFD, ultra- and diafiltration; start, UFD filtrated virus diluted 10× in 10 mM NaP pH 7.4; eluate, fractions from chromatography of start in ~0.5 M NaCl; NFF, normal flow filtration; DF, diafiltration; na, not analyzed; LOD, limit of detection.

[a] From Solvay Pharmaceuticals.

products. The same samples were also analyzed with SRID for comparison. A summary of the results is presented in Table 4. Samples were diluted so that typically two dilutions per sample reached a concentration within the standard curve range. Two replicates of each sample were used and the coefficient of variation (CV) was calculated for each dilution. For most of the samples the results from the two methods corresponded well with each other, although the biosensor measurements showed a significantly lower CV between replicates as well as higher sensitivity than SRID. For a few samples with high HA concentrations (>100 μg/ml), linearity in the dilutions were difficult to achieve. However, this was noted with both the biosensor method and SRID and was therefore thought to derive from the sample rather than from the dilutions as such.

3.6. Trivalent vaccine analysis

Biacore T100 allows samples to be injected over four flow cells in series, with independent sensor surfaces in each flow cell. To analyze trivalent bulk vaccine samples in one experiment, the sensor surfaces in three flow cells were immobilized with HA A/H1N1,

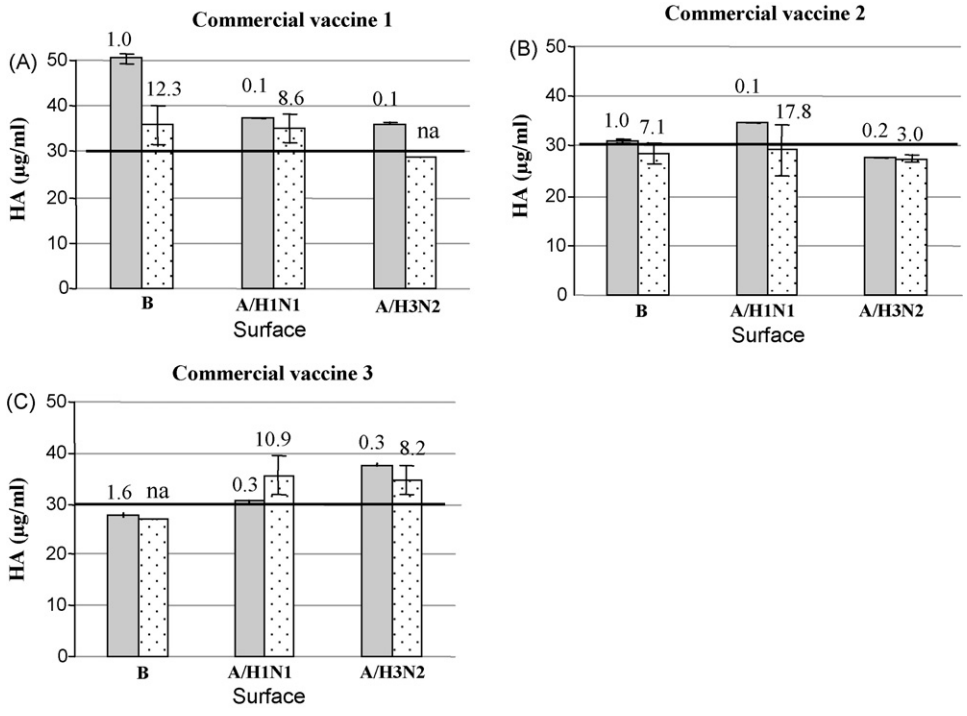

Fig. 7. Analyses of three commercial vaccines, (A–C). The vaccines were quantified using SRID (dotted bar) and biosensor (grey bar). The biosensor analysis was performed on one sensor chip, using three surfaces simultaneously immobilized with B, A/H1N1 and A/H3N2 HA proteins respectively, running the trivalent vaccines over all three surfaces in a single experiment. Coefficient of variation (CV %) of the replicates are presented over each column ($n=2$). The black line represents the specified concentration from the manufacturer of 30 μg/ml of each strain in the vaccine (15 μg per dose). na, not analyzed in replicates.

A/H3N2 and B respectively. Vaccines from three manufacturers were then analyzed using trivalent mixtures of reference antigen and the specific antisera (Fig. 7). The vaccines were also analyzed with SRID (on three separate gels). Again the measurements were similar for SRID and the biosensor method, with higher precision for the biosensor replicates. The total time of analysis was 9 h for the biosensor method including hands on time of 1–2 h. This compared with the total time for the SRID analyses of 22 h, with hands on time of 6–8 h.

4. Discussion

In this paper we present the development and initial results of a novel biosensor method for quantification of HA, utilizing the same reference antigens and sera that are used for SRID, in combination with immobilized HA.

Recombinant HA proteins from different suppliers were tested for immobilization and selected based on immobilization properties, e.g. activity and high enough concentration for direct dilution into immobilization buffer (data not shown). The selected proteins could easily be immobilized in close to neutral (pH 6.5–7.0) buffers, using standard amine coupling procedure. Other immobilization chemistries, coupling through reactive disulfides [29] or aldehyde groups introduced on the protein [30], also resulted in active surfaces that were however slightly less stable over time than those obtained with amine coupling (data not shown). In addition to using recombinant HA on the surface, preliminary studies show that monovalent bulk vaccines (MBV), in particular subunit or split vaccines, can be immobilized and provide similar quantification results (data to be presented elsewhere). MBV would potentially be a more robust source of surface material as the vaccine manufacturers would ensure the supply in their annual production.

The limit of detection of SRID was in our hands found to be ~5 μg/ml, with a quantification range of ~10–30 μg/ml (data not shown), while in the biosensor method the detection limit was estimated as <0.5 μg/ml for all three subtypes with a quantification range of ~1–15 μg/ml. The higher sensitivity of the biosensor method would be valuable in influenza purification process development, where fractions from optimization experiments tend to have low concentrations of virus. Also, crude samples from cell culture supernatants from infected cells may have concentrations of less than 10 μg/ml, at least early on in the infection process. In addition, new types of adjuvant have made it possible for vaccines to be prepared with <7 μg HA/dose [31,32]. For these vaccines batch release of final products would be difficult with SRID.

A downward drift of serum response levels was found during analysis. A biosensor analysis without any response drift is always desirable but is not possible to obtain in practice for many interaction studies, including the one presented here. Instead controls are in such cases included to keep track of surface activity or, as in the present method, calibration curves run several times during the analysis serve as controls. The evaluation software has built in compensation for drift and here prototype software functionality, available for Biacore T100 shortly, was used for further simplification of data evaluation.

For regeneration of the surface, the use of a short (30 s) injection of 50 mM HCl, 0.05% P20 was found to be superior in maintaining surface activity compared to a vast range of other regeneration solutions. As HA is known to undergo irreversible conformational change at low pH [33], subtype specificity for 27 sera of different strains were studied using the short injection of 50 mM HCl, 0.05% Surfactant P20 as regeneration (Fig. 5). All tested sera were subtype specific, e.g. only H3N2 sera bound to recombinant HA H3N2, only B sera bound to B and only H1N1 sera bound to a H1N1 surface, indicating that specificity is maintained using this regeneration. In these experiments the sera were diluted in proportion to titers for SRID, provided by the manufacturer, to take into account differences in serum affinities. Some differences in binding level of sera within a subtype may be due to the fact that the recommended serum dilution for SRID may not be optimal for the biosensor method. In biosensor methods binding of also low affinity antibodies is detected, which may or may not be the case for SRID where the optimal dilution of the serum is that which causes precipitation. With the biosensor method it may therefore be worth exploring the possibility to obtain preliminary quantification with serum earlier in time, before the final serum has matured to high affinity. The subtype specificity results and perhaps more important the high quantification accuracy (Fig. 3, Tables 1 and 4) indicates that regeneration is not affecting analysis performance negatively. Using this regeneration, binding activity sufficient for reliable quantification was maintained for >100 samples/standards for all three subtypes.

Surfactant P20 (Tween 20) was included in all solutions as it is known to help to prevent adsorption of proteins to the flow system without affecting the interaction analysis itself, thus allowing for crude samples to be analyzed. Further, addition of non-ionic detergents were shown to be beneficial against HA aggregation [33].

Strain specificity was briefly explored for H3N2 using two randomly chosen strains, New York and Wyoming. As would be expected due to the genetic difference, the two sera and reference antigens showed different binding behavior towards the other strain (Fig. 6). Highest sensitivity was obtained for the combination where both antigen and serum were Wyoming. This reflects the fact that these reagents are of higher affinity than the New York reagents, resulting in a more effective inhibition of binding to the surface, rather than the fact that the immobilized HA was Wyoming. Different absolute concentrations would be obtained using reference and serum of a different strain than that of the analyzed samples. Still, comparing yield in process development does not always require the absolute concentration. This may be explored further through studies of how differences may correlate depending on how genetically close the strains are.

Sucrose (>1%) and sodium chloride (>0.2 M) did interfere with the measured response in the robustness testing. The response decreased (increasing the calculated HA concentration) with increased sucrose and salt concentration. It is known that at least for salt increasing the concentration significantly above physiological conditions decreases antibody binding to antigens. During vaccine purification, sucrose gradients and chromatography columns are often used producing samples with >40% sucrose and >0.5 M of sodium chloride. However, as the virus concentration in such samples are usually high (>200 μg/ml), the effect of the additives may be avoided in the dilution process.

The seasonal influenza vaccines should contain 15 μg hemagglutinin of each of the three different influenza virus strains: A/H1N1, A/H3N2 and B. In order to analyze the trivalent seasonal flu vaccine bulks and end products with SRID, the vaccine manufacturers have to perform three separate analyses. With the biosensor method it is possible to quantify the three different virus strains in a vaccine in a single experiment (Fig. 7), making it more efficient for batch release of trivalent vaccines. In addition, the biosensor method showed higher recovery and precision in measurements compared with SRID. The higher precision could potentially decrease the need of overfilling vaccine doses.

Taken together, the initial data presented here point to high accuracy and higher sensitivity and precision compared to SRID, in combination with significantly less analysis and hands on time. Expanding the method to a format with immobilized subunits, split

or even whole virus vaccines would open up for a wider use of the quantification method, as well as for basic research such as epitope mapping and kinetic studies.

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