Development of a High-Throughput Screening Platform to Study the Adsorption of Antigens onto Aluminum-Containing Adjuvants

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Received 28 July 2014; revised 2 October 2014; accepted 17 October 2014

Published online 2 December 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24256

ABSTRACT: Aluminum-containing salts are important adjuvants in the formulations of many licensed human vaccines. However, in the early stage of the design of a new vaccine, a thorough understanding of the adsorption mechanisms of an antigen onto an aluminum salt is required. Therefore, we have developed a robust, rapid, and reproducible high-throughput screening (HTS) platform to study the adsorption capacity of aluminum-containing vaccines. The adsorption isotherms on aluminum hydroxide and aluminum phosphate of two model proteins, β-casein, and bovine serum albumin, were evaluated using a liquid handling system, which permitted rapid sample preparation in a small volume without nonspecific adsorption. Highly reproducible adsorption capacities and adsorptive coefficients were estimated based on the Langmuir model. To demonstrate the potential of this HTS platform, we evaluated the adsorption isotherms for two antigens, hepatitis B surface antigen and a pneumococcal serotype polysaccharide conjugated to a protein-D carrier, onto aluminum-containing vaccines at either a constant protein or a constant aluminum concentration. The automated assay enabled the rapid quantification of antigen adsorption with a significant reduction in operator workload and reagent use. This platform should accelerate data acquisition during the early stage of the design of a new vaccine, a thorough understanding of the adsorption mechanisms of an antigen onto an aluminum salt is required.

Keywords: aluminum hydroxide; adsorption; vaccine adjuvants; vaccines; automation; high-throughput screening; adsorption isotherm; adsorptive coefficient; antigen; formulation vaccines

INTRODUCTION

Aluminum-containing adjuvants have remained an important part of many approved human vaccine formulations4–6 since their discovery by Glenny in 1926. The adsorption of an antigen onto an aluminum-containing vaccine is commonly accepted to be an important factor for enhancing the immune response, and the degree of antigen adsorption can affect the immune response. Antigen adsorption onto aluminum-containing vaccines is affected by electrostatic attraction, hydrophobic forces, and phosphate-ligand exchanges. The most frequently used adjuvants in antigen-adsorbed vaccine formulations are aluminum hydroxide (AH) and aluminum phosphate (AP) adjuvants, also known as aluminum oxyhydroxide and aluminum hydroxy-phosphate, respectively. Although both compounds are salts of aluminum, they have different physicochemical properties. The AH adjuvant typically exists in a crystalline form with a point of zero charge (PZC) of approximately 11.4. The AP adjuvant is amorphous and presents a PZC of 4.0–5.0.

Antigen adsorption to aluminum salts reflects an equilibrium between the free antigen in solution and the adsorbed antigen. After a period of equilibration, followed by centrifugation, antigen adsorption is assessed by measuring the concentration of free antigen that remains in the supernatant. The amount of adsorbed antigen is calculated by subtracting the amount of free antigen from the amount of total antigen used. An adsorption isotherm can be evaluated (based on the Langmuir model) using a series of measurements at a constant temperature and a controlled pH, whereby the antigen concentration is titrated against a fixed concentration of adjuvant [i.e., constant aluminum concentration (CAC)] or the adjuvant concentration is titrated against a fixed concentration of antigen [i.e., constant protein-antigen concentration (CPC)]. Hence, the adsorptive properties can be described based on two parameters: the adsorptive capacity, that is, the amount of antigen adsorbed to the solid phase (assumed to be as a monolayer) and the adsorptive coefficient, that is, the affinity of the antigen for the sorbent surface.

The assessment of adsorption isotherms has typically been conducted manually using sample volumes of a few milliliters and hence, this process requires a large amount of antigen and is labor-intensive. High-throughput technologies are less labor-intensive, they can provide more rapid assessments over a wider range of experimental conditions and they use smaller sample volumes thus reducing costs.

In this report, we aimed to develop a robust, rapid, and reproducible high-throughput screening (HTS) platform for the evaluation of adsorption isotherms of model proteins and protein antigens in small volumes. Two different model antigens with different adsorption behaviors were selected for the assay development: bovine serum albumin (BSA) and β-casein. AH has a high-adsorptive capacity for BSA at neutral pH, which involves electrostatic attraction. Although the adsorptive capacity of AH or AP for β-casein has not been reported, this value has been reported for α-casein. We selected β-casein based on the high purity of the commercial product.
(>90% purity in comparison with 70% purity for α-casein), its lower phosphate content, and the higher theoretical adsorptive capacity of AP 1,5 for β-casein compared with lysozyme.5 For further assay development, the following two protein antigens were selected: the well-characterized hepatitis B surface antigen (HBsAg) 1,5,21,22 and a pneumococcal serotype polysaccharide conjugated to a protein-D carrier (PSD-C), that is, the nonlipidated cell–surface lipoprotein of a nontypeable Haemophilus influenzae strain (PSD-C).

MATERIALS AND METHODS

Adsorption Studies of Model Proteins Using a CAC and a CPC

β-Casein (~26 kDa, isoelectric point (IEP): 4.6–5.1) powder was obtained from Sigma–Aldrich (St. Louis, Missouri). BSA was obtained from Merck (Darmstadt, Germany) (~66 kDa, IEP: 5.0). The AP (adjuphos) and AH (alhydrogel) were purchased from Brenntag (Mülheim/Ruhr, Denmark) and were autoclaved. The Al concentration was expressed in μg Al/mL, corresponding to 2.89 μg AH/mL and 4.52 μg AP/mL. The pH of the suspensions of AH/BSA and AP/β-casein was adjusted, with agitation, to 6.1 or 7.0 ± 0.1, respectively, using 100 mM HCl and NaOH.

Nunc polypolyethylene (PP) 96-deep-well plates and microplates were purchased from ThermoFisher Scientific (Waltham, Massachusetts) and ultraflat-transparent 96-well acrylic microtiter 200-μL and 100-μL plates (Costar #3635 and #3679) were purchased from Corning (New York, New York). PP troughs of 100 mL and PP disposable tips were obtained from Tecan (Männedorf, Switzerland). Magnetic stir bars (5 × 2 mm²) were obtained from VWR (Leuven, Belgium). UV-transparent seals (VIEWseal) were obtained from Greiner Bio-one (Kremsmünster, Austria).

The adsorption isotherms were evaluated using a CAC or a CPC. A TECAN Genesis liquid-handling station (Männedorf, Switzerland) was used to determine the adsorption isotherms using 96-deep-well plates.

The CAC adsorption isotherms were determined using a range of protein concentrations (β-casein, 475–750 μg/mL; and BSA, 1800–3000 μg/mL) and 160 μg Al/mL for AP or 1000 μg Al/mL for AH. Ranges of the aluminum and protein concentrations were based on preliminary studies and included the maximum adsorptive capacity for a given aluminum salt/protein combination (data not shown). A sodium-chloride solution was used for β-casein, and water-for-injection was used for BSA to attain final volumes of 250, 300, 400, and 500 μL in the deep wells. Casein solubility depends on the pH and ionic strength of a solution. The addition of sodium chloride increased the casein solubility.22 The CPC adsorption isotherms (525 μg/μL β-casein and 1500 μg/μL BSA) were determined using a range of aluminum–salt concentrations (AP, 210–350 μg Al/mL; and AH, 500–833 μg Al/mL). Sodium chloride and water-for-injection, for β-casein and BSA, respectively, were used to reach the final volume of 500 μL. The formulations were gently stirred using magnetic bars in the microplate for 18 h at room temperature to ensure the homogeneity of the mixtures and avoid sedimentation, using a Vortex Lateral Tumble Stirrer VP 708-CON (V&P Scientific, San Diego, California). Adjuvant-free (protein-only) and protein-free formulations were prepared as controls. The adsorption isotherms on the vial scale (4 mL) were evaluated under the same conditions.

Aluminum salt-free supernatants were obtained after centrifugation (Beckman centrifuge GS-6R, rotor type Swing GH 3.8; Brea, California) at 930 g for 15 min. Liquid transfer from the 96-deep-well plate to the 96-well Costar plate was performed using a Liquidator 96 Manual Benchtop Pipetting System (Bedfordshire, UK). A bicinchoninic acid (BCA) protein assay (Pierce, Rockford, Illinois) was used to determine the protein concentration in the supernatants. The concentration was determined using a standard curve of β-casein (0–200 μg/mL) in 150 mM NaCl or of BSA (0–1000 μg/mL) in water-for-injection. After an incubation of 30 min at 37°C with BCA, samples were centrifuged at 930 g for 2–3 min to remove the bubbles. Absorbance at 562 nm was measured using a plate reader (Varioskan Flash Mode Reader, ThermoFisher Scientific, Massachusetts, USA). For each protein formulation, the buffer-alone signal was subtracted.

To estimate the adsorptive capacity and adsorptive coefficient, the Langmuir model was used.2 The linear regression of the Langmuir equation was obtained by plotting the protein concentration in the supernatants (expressed in milligram) divided by the amount of antigen that was adsorbed (total antigen minus antigen in the supernatant) per milligram of adjuvant (x-axis) against the protein concentration in the supernatants (y-axis). When the R² of the linear regression was higher than 0.99, it was assumed that the Langmuir model described the adsorption isotherm. The adsorptive capacity (Cmax) was calculated from the inverse of the slope of the regression line. The adsorptive coefficient (Ks) was calculated by dividing the slope by the intercept (y-axis) of the regression line.13 To estimate the adsorption isotherms using a CPC, the free protein in the supernatant after centrifugation was normalized to 1 mg Al/mL.

The pH values of the adsorbed formulations were determined using a Cavro Mini Sample Processor (Männedorf, Switzerland) with Gemini software that was customized for pH measurements. The optical density at 320 nm of the supernatant of adjuvant-free (protein-only) solutions was measured in the nonabsorbing regions of the absorption spectra to monitor the presence of aggregates, using UV-transparent microplates and a Varioskan Flash microplate reader.23 The blank signal was subtracted for each protein formulation.

Adsorption Studies of HBsAg onto AH and AP

Purified HBsAg (~3500 kDa, IEP of 4.525; obtained from GlaxoSmithKline) was dissolved in 150 mM NaCl and 10 mM phosphate buffer, pH 6.8. Dilutions of HBsAg were prepared using the same buffer. HBsAg was adsorbed onto AH or AP pre-equilibrated at pH 6.8 or 5.3 ± 0.1, respectively. Solutions for determining the adsorption isotherms with CAC (140 μg Al/mL for AH and 300 μg Al/mL for AP) or CPC (175 μg/mL HBsAg with AH and 350 μg/mL with AP) were prepared in 500 μL in 96-deep-well plates using a TECAN Genesis liquid handling station. The ratios of HBsAg/Al selected were 0.25, 0.5, 0.75, 1, and 1.25 for AH, and 0.5, 0.83, 0.92, 1.0, and 1.17 for AP using both CAC and CPC, as adapted from Hansen et al.18 The adjuvant-free supernatant was obtained by centrifugation of the 96-deep-well plates at 930 g for 15 min. Liquid transfer from the 96-deep-well plates to the Costar plate was performed using a Liquidator 96 Manual Benchtop Pipetting System. The concentration of HBsAg in the supernatant was determined from the optical density at 280 nm that was measured using a plate reader (Varioskan Flash Mode Reader). Measurements

Figure 1. High-throughput screening versus vial scale. Adsorption isotherms (a–c) and Langmuir linearizations (b–d) for the adsorption of BSA by AH (a and b) and β-casein by AP (c and d) at the HTS and vial scale.

Adsorption Studies of Model Proteins Using a CAC

Bovine serum albumin and β-casein were selected as the model proteins to develop the HTS assay for AH and AP, respectively. Preliminary studies showed that adsorption and pH equilibration were achieved after 18 h of incubation with stirring for AH/BSA and AP/β-casein (data not shown). Adsorption of negatively charged BSA (IEP 5.0) by positively charged AH (PZC 11.4) at pH 6.0 ± 0.1 was in agreement with the Langmuir model ($R^2 > 0.99$) (Fig. 1). Adsorption of negatively charged β-casein (IEP 4.6–5.1) by negatively charged AP (PZC 4.5) at pH 7.0 ± 0.1 also fitted the Langmuir model ($R^2 > 0.99$).

Adsorptive capacities and adsorptive coefficients for BSA and β-casein that were derived from the Langmuir equation are shown in Table 1. Using 500 μL, the adsorptive capacities were 2.27 mg/mg and 2.25 mg/mg for BSA/AH and β-casein/AP, respectively. At the vial scale (4 mL) and for BSA adsorption onto AH, adsorptive capacity was 2.28 mg/mg, in agreement with previous estimations, and the adsorptive coefficient was 76 mL/mg ($n = 3$). For β-casein adsorption onto AP, adsorptive capacity was 2.33 mg/mg and the adsorptive coefficient was 142 mL/mg ($n = 4$).

To evaluate the effect of downscaling the assay to microplates, the results obtained using four different formulation volumes (250, 300, 400, and 500 μL) were compared with those obtained using the manually prepared volume (4 mL). The data were highly reproducible for all of the tested volumes. No significant differences ($p > 0.05$) in the adsorptive capacities and coefficients were identified except for the adsorption coefficients for BSA onto AH in volumes of 250 and 500 μL. However, the respective adsorption capacities of 2.32 and 2.27 mg/mg were not different from those evaluated using the other volumes; therefore, the overall differences were considered negligible.

RESULTS

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To evaluate the effects of stirring, pipetting, and centrifugation on protein aggregation and recovery, aluminum-free formulations were evaluated using four volumes (250, 300, 400, and 500 \( \mu \)L). Two protein concentrations were selected, representing the (lowest and highest) limits of protein concentration that have been evaluated. The degree of antigen recovery in aluminum-free formulations was at least 88% for both concentrations, and the median values were 95% for \( \beta \)-casein and 93% for BSA (Supplementary Table 1). The UV–Visible absorbance at 320 nm was determined to evaluate potential particle formation.\(^2\) For both BSA and \( \beta \)-casein, low signals were recorded (Supplementary Table 1). High-protein recoveries and negligible particle formation suggested that no major aggregation/precipitation occurred at the protein concentration and volume ranges examined. Hence, BSA and \( \beta \)-casein adsorption onto an aluminum salt appeared to be unaffected by the use of 250 or 500 \( \mu \)L volumes.

The reproducibility of the adsorption method was evaluated by measuring the CVs for inter-runs and intraruns using the 500 \( \mu \)L volume. The 500 \( \mu \)L volume was selected based on the volume required for the different analyses (200 \( \mu \)L for the pH measurement and 25 \( \mu \)L for the BCA assay). Four runs of the liquid handling system, representing 40 distinct isotherms, were performed over 3 days, with three stock solutions of proteins and three aliquots of the same batch of aluminum salts. The Langmuir equation-derived adsorptive capacities for the 40 adsorption isotherms are shown in Table 1. The inter-run and intrarun CVs of the adsorptive capacity were 1.11% and 2.32%, respectively, for BSA/AH and 2.41% and 4.26%, respectively, for \( \beta \)-casein/\( \beta \)-casein(AP).

### Table 1. Adsorptive Capacities and Coefficients of Model Proteins

<table>
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<tr>
<th>Sorbent</th>
<th>Protein</th>
<th>Volume (( \mu )L)</th>
<th>Adsorptive Capacity (mg/mg Al)</th>
<th>95% Confidence Interval</th>
<th>Adsorptive Coefficient (mL/mg)</th>
<th>95% Confidence Interval</th>
<th>Isotherms</th>
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AH, aluminum hydroxide; AP, aluminum phosphate; BSA, bovine serum albumin.

Adсорption Studies of HBsAg onto AH and AP at a CPC and a CAC

Hepatitis B surface antigen was selected as a model antigen because this antigen is well described in the literature. Preliminary studies showed that the adsorption of HBsAg onto AH and AP had reached equilibration after 2 h of stirring and 16–20 h of incubation (data not shown). The adsorption of negatively charged HBsAg (IEP 4.5) onto positively charged AH at pH 6.8 ± 0.1 was in agreement with the Langmuir model (\( R^2 > 0.99 \)) after normalization of the antigen concentration to 1 mg Al/mL (Fig. 4). Similarly, the adsorption of HBsAg by the negatively charged AP at pH 5.3 ± 0.1 was in agreement with the Langmuir model (\( R^2 > 0.99 \)). Adsorptive capacities and adsorptive coefficients for the adsorption of HBsAg onto AH and AP are given in Table 2 Adsorptive capacities for HBsAg onto AH were 0.9 and 0.85 mg/mg using a CAC or a CPC, respectively. Although the difference in the adsorptive capacities was significant (\( p < 0.05 \)), it was also considered negligible. The adsorptive capacities for HBsAg onto AP were 0.79 mg/mg using either a CAC or a CPC (\( p > 0.05 \)).

To evaluate the effects of stirring, pipetting, and centrifugation on HBsAg aggregation and recovery, aluminum-free formulations were evaluated. As described above, two antigen concentrations (low and high) were selected. The degree of recovery of the aluminum-free formulations was at least 90% (Supplementary Table 1). The absorbance signals at 320 nm were low (Supplementary Table 1). The absorbance signals at 320 nm were also considered negligible. The adsorptive capacities for HBsAg onto AP were 0.79 mg/mg using either a CAC or a CPC (\( p > 0.05 \)).

Adsortion Studies of PSD-C onto AP at a CPC and a CAC

Pneumococcal polysaccharide conjugated to a protein-D carrier was selected as the second model antigen. Preliminary studies showed that the adsorption of PSD-C onto AP had reached equilibration after 2 h of stirring (data not shown). The adsorption of PSD-C onto AP was in agreement with the Langmuir model (\( R^2 > 0.99 \)) at pH 5.2 ± 0.1 (Fig. 5). The adsorptive capacities (0.19 and 0.18 mg/mg for the CAC and CPC, respectively) and adsorptive coefficients (107 and 73 mL/mg for the CAC and CPC, respectively) of PSD-C onto AP were described further in Table 2 These respective parameters were not significantly different (\( p > 0.05 \)) when estimated using CAC- or CPC-derived adsorption isotherms. A high variability of the adsorptive coefficients was observed.

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Figure 2. Adsorption of BSA onto AH. CAC (black square) and CPC (open square) adsorption isotherms (a) and Langmuir linearizations (b) for the adsorption of BSA by AH.

Figure 3. Adsorption of β-casein onto AP. CAC (black square) and CPC (open square) adsorption isotherms (a) and Langmuir linearizations (b) for the adsorption of β-casein by AP.

Table 2. Adsorptive Capacities and Coefficient of Model Proteins and Antigens

<table>
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<tr>
<th>Protein</th>
<th>Sorbent</th>
<th>Type</th>
<th>Volume (μL)</th>
<th>Adsorptive Capacity (mg/mg Al)</th>
<th>95% Confidence Interval</th>
<th>Adsorptive Coefficient (mL/mg)</th>
<th>95% Confidence Interval</th>
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</table>

BSA, bovine serum albumin; PSD-C, pneumococcal polysaccharide conjugated to a protein-D carrier; AH, aluminum hydroxide; AP, aluminum phosphate; HBsAg, hepatitis B surface antigen.

The evaluation of the aluminum-free preparations showed no detrimental effect of the method on protein recovery (Supplementary Table 1).

DISCUSSION

High-throughput screening technologies are increasingly integrated into the formulation development process for biopharmaceuticals.19,26,28,29 To the best of our knowledge, only one paper has reported the use of HTS methods to evaluate the structural and chemical stability of an antigen adsorbed onto an aluminum-containing adjuvant.26 However, this paper did not describe the preparation of aluminum-containing adjuvanted vaccines using an HTS platform and therefore did not consider utilizing small volumes in comparison with the classical approach. Because adsorption isotherms are largely used to characterize the mechanisms by which proteins are adsorbed by aluminum-containing adjuvants,9,13–15 our study aimed to use the HTS platform to decrease material consumption and the work load while permitting a better approach to evaluating the mechanisms of adsorption and further investigating the effect of excipients on adsorption.

In this report, we described a rapid, robust, and reproducible HTS platform that is suitable for assessing adsorption isotherms in microplates. In downscaling from 4-mL vials to microplates, nonspecific adsorption could occur because...
the surface area-to-volume ratio is increased. Moreover, the interaction of the hydrophobic core of a molecule with a hydrophobic (container wall) surface can lead to the denaturation, self-association, and aggregation of the molecule.30 However, in our study, nonspecific adsorption was not detected, as demonstrated by the high recoveries and low aggregation for all the proteins evaluated.

Adsorption isotherms are generally performed by titrating the concentration of a protein against a CAC.9,13–15 However, in the development of a formulation of a new aluminum-containing adjuvanted vaccine, it is typically more appropriate to maintain a fixed concentration of the antigen (determined by preliminary preclinical studies) and vary the adjuvant concentration. We have demonstrated that the adsorption isotherms estimated using a CPC for BSA, $\beta$-casein HBsAg, and PSD-C were in agreement with the Langmuir model. Moreover, because the estimates obtained for the adsorptive capacities and adsorptive coefficients using a CPC or a CAC were very similar, we concluded that both approaches can be used to study the adsorption of antigens onto aluminum-containing adjuvants.

In this report, two model proteins were selected based on their different adsorption behaviors with aluminum salts. Hem and coworkers9,15,21 have demonstrated that electrostatic forces contribute to the adsorption of negatively charged BSA onto positively charged AH at a neutral pH. The adsorptive capacities of BSA onto AH that were estimated in our study are in agreement with those of previous studies.14,31,32 $\beta$-Casein, a phosphoprotein, contains five phosphate groups.33,34 According to our results, the adsorption of $\beta$-casein by AP at a pH of 7.0 occurred in spite of the electrostatic repulsion between

Figure 4. Adsorption of HBsAg onto AH and AP. CAC (black square) and CPC (open square) adsorption isotherms (a–c) and Langmuir linearizations (b–d) for the adsorption of HBsAg by AH (a and b) and AP (c and d).

Figure 5. Adsorption of PSD-C onto AP. CAC (black square) and CPC (open square) adsorption isotherms (a) and Langmuir linearizations (b) for the adsorption of PSD-C by AP.
negatively charged β-casein and negatively charged AP. In contrast to electrostatic interactions, phosphate–ligand exchange is not affected by the relationship between the pH of the vaccine and the IEP of the antigen or the PZC of the aluminum-containing adjuvant. Hence, phosphate–ligand exchange may be the predominant mechanism for the adsorption of β-casein onto aluminum-containing adjuvants.

Hepatitis B surface antigen particles are composed of host cell-derived lipids and up to three virus-encoded glycoproteins, which form 22-nm spherical particles. Phospholipids account for the major fraction of the lipids of the HBsAg particles. Hem and coworkers have demonstrated that phosphate–ligand exchanges contribute to the adsorption of negatively charged HBsAg by positively charged AH at neutral pH, in addition to electrostatic forces. Our estimates for the adsorptive capacities of HBsAg adsorbed onto AH are lower than the previously published adsorptive capacities, most likely because of the presence of phosphate residues in the HBsAg buffer used in marketed vaccines. For HBsAg adsorption onto AP, our estimates of the adsorption capacities were similar to previously published results obtained using phosphate-treated AH. Phosphate–ligand exchange has been found to be related both to the number of phosphate groups on the antigen and the number of hydroxyl groups on the aluminum-containing adjuvants. Therefore, the probability of antigen adsorption onto AP via ligand exchange is lower than that for AH because of the replacement of hydroxyl groups by phosphate groups.

The pneumococcal serotype polysaccharide in PSD-C is conjugated to carrier protein-D. Typically, polysaccharides are conjugated to carrier proteins to enhance an antigen’s immunogenicity in children. Carrier proteins are used to induce helper T-cell responses and to provide bystander T cell-dependent immunity against the polysaccharides. The adsorptive capacity onto AP for PSD-C (0.19 mg/mg at CAC) was low compared with that of β-casein (2.1 mg/mg) and HBsAg (0.8 mg/mg). In addition to the negative charge of the PSD-C complex at pH 7.0, the decrease in adsorptive capacity can be explained by the fact that polysaccharide can hinder the interaction of the protein and the aluminum salt.

Several other factors can also affect adsorptive capacities, such as the nature of the adjuvant (PZC), the antigen’s molecular weight, conformation and IEP, the adsorption mechanisms, and the adsorption conditions (e.g., sodium chloride, phosphate buffer, and pH). Previous studies on the adsorption of proteins onto aluminum-containing adjuvants showed that proteins that were adsorbed via weak interactions, such as electrostatic and/or hydrophobic attractive forces (i.e., lysozyme and BSA), have relatively low-adsorptive coefficient values. Furthermore, it has been demonstrated that the adsorptive coefficient associated with phosphate–ligand exchange is related to both the degree of phosphorylation of the antigen and the number of surface hydroxyl groups on the aluminum-containing adjuvant. Indeed, proteins that are adsorbed via ligand exchange and that contain few phosphate groups exhibit intermediate adsorptive coefficients (i.e., dephosphorylated α-casein and ovalbumin, which contain zero to two phosphate groups). If the number of phosphate groups increases, proteins show a higher adsorptive coefficient (i.e., α-casein, which contains eight phosphate groups). A high molecular weight can also lead to a high-adsorptive coefficient of a protein, such as fibrinogen (~330,000 Da), because of its large negative region.

The estimates of adsorption capacities were highly reproducible using the HTS method, as indicated by CVs of less than 5%. However, the estimates of the adsorptive coefficients were more variable. The adsorptive capacity for an antigen onto an aluminum-containing adjuvant has been frequently reported, whereas the adsorptive coefficient (or affinity) has not been. When reported, the variation in the adsorptive coefficients for different types of proteins that bind via the same adsorption mechanism and to the same kind of adjuvants is more than several orders of magnitude. Indeed, in the Langmuir model, the adsorptive coefficient is calculated based on only one measurement, the intercept.

Understanding of the adsorption mechanisms of a specific antigen is required for the development of a new vaccine product, and this knowledge must extend from the early stages of the process (formulation and manufacture) to the administration of the vaccine. Indeed, it has been demonstrated that adsorption through ligand exchange affects the elution of antigens from the aluminum-containing adjuvants in the interstitial fluid at the injection site. The degree of antigen adsorption by aluminum-containing adjuvants can affect the immune response. The mechanism of action of aluminum-containing adjuvants is to potentiate an immune response by retaining the antigen at a high concentration in situ and inducing a local inflammatory response by recruiting dendritic cells. However, antigen adsorption that is too strong may interfere with the immune response by affecting antigen processing in the antigen-presenting cells. Furthermore, the adsorption coefficient can also be used to predict potential competition between two or more antigens in a multivalent vaccine, such that the protein with the higher adsorption coefficient can displace an adsorbed protein with a lower adsorption coefficient. Moreover, the adsorption capacity can also be useful in determining the consistency of aluminum-containing adjuvant batches during production. Finally, the HTS method could be used to assess optimal desorption conditions of antigens adsorbed onto aluminum salts without denaturation by screening buffers or desorption agents.

**CONCLUSIONS**

The HTS platform can be used to estimate the adsorption isotherms for antigen aluminum-containing adjuvants using titrations of either fixed aluminum–salt or protein concentrations in microplates. No detrimental effects from downscaling were observed, such as nonspecific adsorption. Using the platform, we were able to quantitatively and reproducibly determine the adsorptive capacities and adsorptive coefficients of AH and AP for two model proteins, BSA and β-casein, and the corresponding values for the antigens, HBsAg adsorbing onto AH and AP and the polysaccharide protein-conjugate antigen PSD-C adsorbing onto AP. The HTS platform has the potential to be incorporated into the vaccine development process as a robust, rapid, and reproducible method to obtain adsorption isotherms.

Incorporating the HTS platform into the vaccine formulation process could facilitate a better understanding of the mechanisms governing the interactions between an antigen adsorbed onto an aluminum-containing adjuvant, the effect that changes
in formulation could have on the antigen adsorption properties and the assessment of the batch-to-batch consistency of aluminum-containing adjuvants.

Acknowledgments
The authors thank C. Dekeyser for fruitful discussions, D. Tassin for technical assistance with the automated system and the Liquid Class development, C. Van Loo for the CAC isotherms of PSD-C and HBsAg experiments and F. Renaud for the statistical analysis. The authors would like to acknowledge M. Morgan (MG Science Communications) for critical review of the manuscript and Ulrike Krause for publication management. Synflorix is a trade mark of the GlaxoSmithKline group of companies.

This work was funded by GlaxoSmithKline Biologicals S.A., under a Cooperative Research and Development Agreement with the Université catholique de Louvain.

All authors participated in the design or implementation or analysis, and interpretation of the study results. All authors were involved in drafting the manuscript or revising it critically for important intellectual content. All authors had full access to the data and approved the manuscript before it was submitted by the corresponding author.

The authors declare the following conflicts: Nicolas Moniotte, Frédéric Mathot, and Dominique Lemoine are employees of the GlaxoSmithKline group of companies. Dominique Lemoine owns shares of GSK. The other authors declare no conflicts.

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