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Hepatitis B Surface Antigen Particle Purification by Immunoaffinity Chromatography Based on CDI-CB.Hep-1 mAb Monolithic Supports

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Abstract

Bead matrices have been used in affinity chromatography to purify molecules in multiple applications. For instance, the hepatitis B surface antigen (HBsAg) is one of the molecules purified by this technique for human vaccine development programs. However, the use of monolithic supports have emerged as the advantageous choice for affinity chromatography based on convective mass transfer, a high number of channels, and low backpressures at high flow rates. For this reason, several experiments were conducted to determine the suitability of CB.Hep-1 monoclonal antibody (mAb) immunosorbent developed on carboxyimidazole (CDI)-monolithic supports (ligand concentrations: 0.5, 1.0, and 7.0 mg/mL) for HBsAg particle purification.

Key results from this study show the highest amounts of HBsAg adsorbed (3059.31 ± 865.71 µg HBsAg/mL immunosorbent, $n=2$), and HBsAg eluted (2884.50 ± 541.01 µg HBsAg/mL immunosorbent, $n=2$), were estimated in the 1.0 mg/mL-CDI-CB. Hep-1 mAb monolithic support immunosorbents. In addition, the ligand leakage was always <3 ng mAb/µg HBsAg (approved limit) in the 1.0 mg/mL-CDI-CB.Hep-1 mAb immunosorbents. Experiments also evidenced the high purity and molecular homogeneity of purified HBsAg particles (<95%) across 20 purification cycles. Therefore, the ligand concentration could be reduced up to 1.0 mg/mL, which would enable a notable decrease in the mAb amount required for vaccine manufacturing, as compared to bead matrices (4.0 mg/mL). This study demonstrated that CDI-CB.Hep-1 mAb monolithic support immunosorbents are best suited for assessing the large-scale purification performance of HBsAg particles for human vaccine development programs at low ligand concentration and high flow rates.

Introduction

The hepatitis B disease is a serious pathology caused by the hepatitis B virus (HBV). For some unknown reasons, HBV infection eventually disappears in some carriers. Nevertheless, for others, HBV infection of the liver becomes chronic, causing fibrosis, failure, cancer, cirrhosis, and also death. In epidemiology terms, it is currently estimated that two billion people have been infected with HBV worldwide. Of these, about 400 million have become chronically infected with HBV, and one million people die every year by complications of this pathology.[1, 2]

The best way to prevent HBV infection and its complications is by getting vaccinated to acquire a long-term protection. Regarding availability of vaccines against hepatitis B, there are several commercially available recombinant hepatitis B vaccines such as: Engerix-B (GlaxoSmithKline); Recombivax HB (Merck & Co.); Twinrix, hepatitis A/B (GlaxoSmithKline); Comvax, hepatitis B/haemophilus b–meningococcal protein conjugate (Merck); and Pediarix, hepatitis B/diphtheria/tetanus/pertussis/poliomyelitis (GlaxoSmithKline).[3, 4] The Cuban Genetic Engineering and Biotechnology Center (CIGB) is also a well-recognized, worldwide producer of this type of vaccine: Heberbiovac HB® (hepatitis B), Trivac HB® (hepatitis B/diphtheria/tetanus/pertussis), and Heberpenta® (hepatitis B/diphtheria/tetanus/pertussis/haemophilus influenzae type b).[5, 6]

All of these recombinant hepatitis B vaccines were created through the genetic engineering of DNA by inserting a segment of viral genetic material into yeast, insect, or mammalian cells that codify HBsAg particles. Recombinant HBsAg particles constitute the active pharmaceutical ingredient (or drug substance) of these vaccines against hepatitis B. Even though the aforementioned hepatitis B vaccines are relatively similar, some process incomparabilities have been observed due to the different methods used to obtain purified HBsAg particles. For instance, the HBsAg particles produced by CIGB have been always purified by a specific combination of steps, the most important one being an immunoaffinity chromatography (IAC) step based on an agarose bead matrix.[7, 8]

The immunosorbents employed in the IAC technique described by Valdés *et al*.[9,10] and Hernández *et al*.[11] were performed by the random attachment of CB.Hep-1 monoclonal antibody (mAb) to Sepharose CL-4B activated with cyanogen bromide (BrCN). This activation procedure has shown a fairly high degree of stability and biological inertness of supports. However, it also has some disadvantages including: BrCN toxicity; a coupling reaction influenced by temperature, initial carbonate concentration, and duration of activation reaction; minimal stability of active groups at basic conditions; a pore size almost too small to handle HBsAg particles; the need for a relatively high ligand concentration (≥4.0 mg/mL) with low strength and flow rates; and the activation procedure lacks a well-defined end-point.

To radically improve HBsAg particle purification efficiency, monolith chromatographic supports have been used for their higher: mechanical resistance, convective mass transfer, number of channels, and capacities for adsorption and elution of large molecules (*e.g.*, viruses and plasmid DNA).[12-14] Monolithic supports are disks or tubular structures characterized by macro and mesopores that provide high porosity, numerous interconnected channels, and a moderately high available surface area for interaction with and among analytes. Fortunately, the backbone of monolithic supports is composed of either an organic or inorganic substrate, and thus it can be easily chemically functionalized for specific applications such as IAC.[15,16]

Despite such remarkable advantages, reports on the use of monolithic supports in the purification of HBsAg particles by IAC are non-existent in the scientific literature. Therefore, the main subject of this study was to explore the suitability of carboxyimidazole (CDI)-monolithic support-based immunosorbents for HBsAg particle purification. The data created can be used to scale-up the hepatitis B surface antigen purification process.

Materials and Methods

Source of CB.Hep-1 mAb (Ligand)

The SP2/0-Ag14-derived hybridoma CB.Hep-1 was obtained using a BALB/c mouse immunized with HBsAg particles (from a person chronically infected with the hepatitis B virus) and emulsified in Freund adjuvant (Sigma-Aldrich).[17] Hybridoma cells were isolated and then cultivated in 1 L spinner flasks (starting with 3×10^5 cells/mL) using Gibco® (Thermo Fisher Scientific) RPMI-1640 cell culture medium and fetal bovine serum (supplemented at 8%), 2 mM L-glutamine, 1 mM sodium pyruvate, and 17 mM sodium bicarbonate, maintained at 37°C in 5% CO_2 atmosphere. Cells were adapted to a serum-free medium and then used to inoculate a 50 L Appliflex single-use bag rocker bioreactor (Applikon Biotechnology) for CB.Hep-1 mAb production.[18]

Purification of CB.Hep-1 mAb

Bioreactor supernatant was continuously harvested, centrifuged at 1500×*g*, concentrated using a 30 kDa SARTOFLOW® Alpha cartridge (Sartorius) and filtered with 0.22 µm filtration membranes. It was then purified by protein A Sepharose® Fast Flow (PASFF, GE Healthcare) IAC using 150 mM phosphate-buffered saline (PBS) at pH 8.0 as an adsorption buffer and 100 mM citric acid at pH 3.0 as the elution buffer.[18] An XK50/30 column (GE Healthcare) was operated at a linear flow rate of 100 cm/h. Extensive washings with 150 mM PBS at pH 8.0 were done to remove other contaminants. Subsequently, the purified CB.Hep-1 mAb in 100 mM citric acid, pH 3.0 and 4°C, was performed as a virus inactivation step followed by an ultrafiltration step to concentrate samples. Afterward, the sample buffer was exchanged to 20 mM Tris/150 mM NaCl, pH 7.6, by size-exclusion chromatography (SEC) using coarse Sephadex G-25 gel filtration resin (GE Healthcare) in an XK50/30 column operated at 130 cm/h, and the resulting material was filtered under sterile conditions with a 0.22 µm membrane filter.

Source of HBsAg Particles

HBsAg particles were produced by the fermentation of *Pichia pastoris* yeast cells. Cells were harvested by centrifugation and disrupted on a DynoMill KDL disintegrator (Willy A. Bachofen). The homogenate underwent acid precipitation and as well as centrifugation at 10,000×*g* for 30 min. Supernatant was placed in contact with the Celite® Hyflo Supercel (Sigma-Aldrich) filtration aid and equilibrated to the same pH under continuous stirring. Following adsorption, the filtration aid was separated by centrifugation. The matrix was washed with two Supercel volumes of 2 M potassium thiocyanate solution and HBsAg particles were eluted with 20 mM Tris-HCl/3 mM EDTA/100 g/L sucrose, pH 8.2. HBsAg particles were then purified by a series of well-established steps including agarose-based IAC, ion-exchange chromatography (IEC), and SEC.[7,8]

Activation of Monolithic Supports

Activated CIMac™ monolithic analytical columns (BIA Separations) with a pore size of 700 nm, 5.2 mm inner diameter (ID), 5 mm length, and 106 µL of volume were used. The CDI chemistry was used to activate monolithic supports following information published by Pfaunmiller *et al*.[15]

Immobilization of CB.Hep-1 mAb on CDI-Monolithic Supports

The CB.Hep-1 mAb immobilization on CDI-monolithic supports was performed using CB.Hep-1 mAb dissolved in 0.1 M borate buffer, at pH 8.0, containing 1 M NaCl. In all cases, columns were first washed with 10 column volumes (CVs) of water and with 20 CVs of the immobilization buffer. Next, 10 CVs of respective CB.Hep-1 mAb amounts dissolved in the immobilization buffer were cycled through the columns at 50 µL/min. Each immobilization system was set to cycle

up to 24 h at 25°C. After mAb immobilization, the remaining CDI-free activated groups were blocked with a 2 M ethanolamine solution, pH 8.5, by washing each column with 10 CVs at room temperature (22±2°C). The compendium of CDI-monolith support columns manufactured to be studied in this research were CIMac CDI-CB.Hep-1 mAb immunosorbents at: (1) 0.5 mg/mL (*n*=2); (2) 1.0 mg/mL (*n*=2); and (3) 7.0 mg/mL (*n*=1).

Quantification of CB.Hep-1 mAb on CDI-Monolithic Supports

The amount of immobilized CB.Hep-1 mAb on monolithic supports was determined by the quantification of mAb in the non-immobilized fractions using CIMac protein G analytical columns. The analyses were performed on the Agilent 1200 Series HPLC system coupled with a multiple wavelength detector. Samples were diluted with 150 mM PBS, pH 7.6, and injected (100 µL sample loop) onto 106 µL protein G columns. The loading mobile phase was 150 mM PBS, pH 7.6, while the elution was performed by 100 mM glycine buffer, pH 2.0. The flow rate used was 1 mL/min and the detection wavelength was set to 280 nm. CB.Hep-1 mAb amount quantification was performed using a calibration curve standardized with that of pure CB.Hep-1 mAb samples.

First Chromatography Assessment of CB.Hep-1 mAb CDI-Monolithic Support Immunosorbents

This assessment was done with immunosorbents performed at 0.5, 1.0, and 7.0 mg/mL of CB.Hep-1 mAb (ligand) concentrations. The applied material was made up of 1 mg of purified HBsAg particles diluted in 20 mL of column equilibration buffer containing 20 mM Tris/3 mM EDTA/0.5 M NaCl, pH 7.4. Next, after antigen application, the columns were washed with 10 CVs of equilibration buffer and the elution was carried out using 20 mM Tris/3 mM EDTA/0.5 M NaCl/3 M KSCN, pH 7.4. All chromatography steps were done at a flow rate of 1 mL/min in a LaChrom semipreparative HPLC system (Merck-Hitachi) with an L-7400 UV detector and L-7100 pump. Sample absorbance was always measured at 280 nm and data were acquired and processed by Biochrom software developed by the CIGB (purification cycle=1).

Second Chromatography Assessment of CB.Hep-1 mAb CDI-Monolithic Support Immunosorbents

While this study was done with immunosorbents using the same ligand concentrations (0.5, 1.0, and 7.0 mg/mL), the applied material was always non-purified HBsAg particles (1 mg of HBsAg diluted in 20 mL of the samples obtained through IEC). The chromatography conditions used were the same as those described in the first chromatography assessment. After each purification cycle, columns were regenerated using: 20 mL of 0.1 M Tris-HCl/0.5 M NaCl/0.1 M NaAc, pH 8.5; 20 mL of purified H_2O; 20 mL of 0.1 M Tris-HCl/0.5 M NaCl/0.1 M NaAc, pH 4.0; and 20 mL of purified H_2O, respectively (purification cycles=5).

Third Chromatography Assessment of CB.Hep-1 mAb CDI-Monolithic Support Immunosorbents

This study was done with an immunosorbent using a ligand concentration of 1.0 mg/mL. The applied material was always a non-purified HBsAg (1 mg of HBsAg diluted in 20 mL of the samples obtained through IEC). The chromatography conditions and regeneration used were the same as was described in the first and second chromatography assessments, respectively. In this study, 20 purification cycles were done primarily to analyze the purity level and molecular homogeneity of the purified HBsAg particles and the ligand leakage.

Protein Quantification

Protein quantification was performed following the procedure described by Lowry *et al*.[19] The calibration curve ranged from 10–100 µg/mL. The absorbance was measured at 730 nm in an Ultrospec UV/Visible spectrophotometer (GE Healthcare). Purified samples were also quantified by measuring absorbance at 280 nm and using molar extinction coefficients (5 for HBsAg particle and 1.37 for CB.Hep-1 mAb).

SDS-PAGE and Size-Exclusion (SE)-HPLC to Determine CB.Hep-1 mAb and HBsAg Particle Purity and Molecular Homogeneity

The identity pattern and purity of CB.Hep-1 mAb and HBsAg particles were analyzed by gel electrophoresis on a 12.5% (w/v) SDS-PAGE followed by Coomassie® brilliant blue R-250 (Bio-Rad Laboratories) staining, as described by Laemmli.[20] Molecular homogeneity of CB.Hep-1 mAb was estimated by SE-HPLC using a TSKgel G3000PW (3000 mm/7.5 mm ID, Tosoh Bioscience) column. The chromatographic mobile phase was 150 mM PBS, pH 7.0, and 100 µg of sample dissolved in 150 mM PBS, pH 7.0, directly applied into the system. The volumetric flow rate employed was 200 µL/min and the absorbance was measured at 226 nm. The molecular homogeneity of HBsAg particles was estimated using a TSKgel G6000PW (6000 mm/7.5 mm ID, Tosoh Bioscience) SE-HPLC column. The chromatographic mobile phase was 150 mM PBS, pH 7.0, and 100 µg of sample dissolved in 150 mM PBS, pH 7.0, applied into the system. The volumetric flow rate employed was 600 µL/min and the absorbance was measured at 226 nm.

Enzyme-Linked Immunosorbent Assay (ELISA) for CB.Hep-1 mAb Quantification in Unpurified Samples

MaxiSorp microtiter plates (Thermo Fisher Scientific) were coated with 100 µg/mL of HBsAg particles in carbonate/bicarbonate buffer, pH 9.6. After incubation, plates were washed. All samples were diluted in 150 mM PBS, pH 7.6/0.2% of bovine serum albumin/0.005% Tween 20 and incubated again for 20 min at 50°C. Subsequently, wells were washed five times and incubated with 100 µL/well of a goat anti-mouse gamma immunoglobulin (IgG) horseradish peroxidase conjugate for 20 min at 50°C.

Finally, plates were washed and the reaction was revealed using *o*-Phenylenediamine as substrate and 0.015% H_2O_2 in citrate buffer, pH 5.0. The reaction was stopped by adding 50 mL of 2 M H_2SO_4 and immediately measured at 492 nm using a Multiskan ELISA reader (Thermo Fisher Scientific).

ELISA to Determine CB.Hep-1 mAb Released from Immunosorbents

Costar high-binding plates (Corning) were coated with a sheep anti-mouse polyclonal IgG overnight at 4°C. Plates were blocked for 30 min at 37°C. Next, wells were washed and eluted samples from immunosorbents were added and incubated for 3 h at 37°C with 1% non-fat milk/150 mM PBS, pH 8.0. After washing, plates were incubated with 100 µL/well of a goat anti-mouse polyclonal IgG-horseradish peroxidase conjugate (Sigma-Aldrich). The reaction was then revealed using 100 µL/well of 0.05% *o*-Phenylenediamine and 0.015% H_2O_2 in citrate buffer, pH 5.0, and stopped with 50 µL/well of 1.25 M H_2SO_4. The absorbance was also measured in a Multiskan ELISA reader using a 492 nm filter.[21]

Statistical Analysis

Microsoft Excel software was used as the tool for mathematical analysis and the Statgraphics® Centurion XV v. 15.2.06 (Statistical Technologies) for statistical analysis. The Student's *t*-test and one-way analysis of variance (ANOVA) tests were used to compare the adsorbed and eluted amount of HBsAg particle results using 0.05 as the confidence level. The Duncan's test was employed to discriminate among variables with statistical differences.

Results and Discussion

Hepatitis B vaccines are based on recombinant HBsAg virus-like particles. The HBV envelope consists of three glycoproteins called L, M, and S-termed HBsAg particles, which are translation products of a single open-reading frame in the HBV DNA (pre-S1, pre-S2, S segments). In natural infections, the most abundant protein is the protein encoded by the S segment, which exhibits a lipoprotein-like structure with more than 70 S-protein molecules in association with various types of lipids. Yeast transformants express HBsAg particles with the same morphology as those observed in the serum of individuals chronically infected with HBV. Examination of HBsAg particles expressed in yeasts by electron microscopy has revealed an average particle size of 22 nm of unglycosylated forms of S protein. As it has been broadly reported, these HBsAg particles constitute an excellent tool for inducing a long-term protective immune response against the HBV infection.[3,4]

As a rule, the expression of HBsAg particles in yeasts such as *Saccharomyces cerevisiae* occurs intracellularly. Thus, yeast cells have to be disrupted after the fermentation process and then clarified prior to purification of HBsAg particles by several techniques such as IEC, ultrafiltration, isopycnic density centrifugation, and SEC. In general, the applied purification processes yield HBsAg particles with ≥95% purity that are biologically active, immunologically efficacious, and well-tolerated by vaccinated people.[3-8] Simultaneously, researchers from CIGB also demonstrated the ability of *P. pastoris* yeast-based technology for large-scale production of HBsAg particles. In the reported purification process, the clarification of mechanically disrupted yeast cells by acid precipitation rendered HBsAg particles with very low purity, which then increased the adsorption/desorption from diatomaceous earth matrix to allow the purification of the protein by IAC, IEC, and SEC to eliminate or reduce non-particulated forms of HBsAg particles, and lowered the carbohydrates and lipids to acceptable levels for human use.[7,8] However, two problems associated with the use of IAC have been the relative low recovery (approximately 50%) and the small number of purification cycles (<19). Authors speculated that both problems are strongly linked to characteristics of the non-purified material, structural characteristics of HBsAg particles (particle size), HBsAg particle nanomolar association constant with the CB.Hep-1 mAb (ligand), and finally with properties of Sepharose CL-4B (relative small pore size) which does not favor the diffusion of the antigen into the beads.

Therefore, in order to overcome these drawbacks, CDI-monolithic supports were explored in this study because they have very short diffusion distances, providing multiple pathways for solute dispersion. This type of support allows the target analyte to enter in one channel and exit through any of six or more different venues. Moreover, the high degree of interconnectivity confers advantages to achieve high flow rates.[12-16] The chromatographic efficiency of CDI-group monolithic supports was investigated here because this chemical activation method also involves the attachment of proteins by amine groups randomly and non-oriented to the supports like the BrCN activation method.[12-16,22-24] One reasoning behind this target is that some antibody molecules will be unable to recognize the HBsAg particles by their immobilization on the support by the antigen recognition site, which may favor a decrease in the strength of association with the antigen molecule with the repeated epitope.

For such HBsAg particle separation analysis, three CDI-monolithic supports (ligand concentrations: 0.5, 1.0, and 7.0 mg/mL) were challenged firstly with previously purified HBsAg particles. This initial study was done to avoid the influence of the non-purified samples in the initial deductions over the efficiency of CDI-immunosorbents. Results of this initial experiment are shown in **Table 1** on the following page. As it can be noted, the antigen adsorption capacity increased with the decrease of the ligand concentration from 964.3 down to 6400 µg HBsAg/mg mAb. However, due to high ligand concentration, the higher adsorbed antigen amount was estimated in the

TABLE 1. Summary of the HBsAg particle adsorption and elution data of CDI-CB.Hep-1 mAb monolithic support immunosorbents immobilized at different ligand concentrations, and measured during the application of purified HBsAg particles. The HBsAg particles were always applied in 20 mM Tris/3 mM EDTA/0.5 M NaCl, pH 7.4, and eluted with 20 mM Tris/3 mM EDTA/0.5 M NaCl/3 M KSCN, pH 7.4.

Ligand Concentration (mg mAb/mL)	Antigen Adsorption Efficiency (%)	Antigen Adsorption Capacity (µg HBsAg/mg mAb)	Antigen Adsorbed Amount (µg HBsAg/mL)	Antigen Elution Efficiency (%)	Antigen Elution Capacity (µg HBsAg/mg mAb)	Antigen Eluted Amount (µg HBsAg/mL)
7.0	67.5	964.3	6750.1	35.6	342.9	2400.3
1.0	32.0	3200.0	3200.0	100.8	3225.0	3225.0
0.5	32.5	6400.0	3200.0	100.8	6450.0	3225.0

7.0 mg/mL CDI-immunosorbent (6750.1 µg HBsAg/mL of immunosorbent). It was 2.1-fold higher that in the rest of the immunosorbents. On the other hand, even though the differences in antigen adsorption capacity observed between the 1.0 and 0.5 mg/mL immunosorbents were corroborated, the antigen adsorption amount was similar with both immunosorbents (3200 µg HBsAg/mL). In contrast, the lowest antigen elution capacity (342.9 µg HBsAg/mg mAb) was detected in the 7.0 mg/mL immunosorbent (the highest ligand concentration). As a consequence, only 2400.3 µg HBsAg/mL of immunosorbent was measured in this fraction, which corresponded with 74% of antigen eluted from 0.5 and 1.0 mg/mL immunosorbents (3225.0 µg HBsAg/mL). Interestingly, the elution efficiency [(eluted amount/adsorbed amount)×100] calculated in low ligand concentration-immunosorbents was 100.8%, evidencing no retention of the HBsAg particles in the support.

Authors theorized that the lowest elution capacity results observed in 7.0 mg/mL CDI-immunosorbent could be provoked by the high ligand concentration (high avidity), which is an inconvenience for the strong association constant between HBsAg particles and the CB.Hep-1 mAb molecules. The association constant among these two molecules is in the order of 10^9–10^{10} M^{-1}[25], but in the chromatography the avidity could be even higher due to the presence of a repeated epitope in the HBsAg particles (70-fold).

Therefore, these preliminary results are considered promising for assessing the application of non-purified HBsAg particles in low-ligand concentrations of CDI-immunosorbents, especially for potentially drastic reductions in CB.Hep-1 mAb consumption, and for eliminating the verified HBsAg particle retention phenomenon in BrCN-bead immunosorbents (about 30%). Thus, these results confirmed that column retention of HBsAg particles was favored at the higher ligand concentration, evidencing that the attachment of mAb with high affinity constants on CDI-monolithic support should not be the best choice for the purification of HBsAg particles with the repeat epitope.

Taking into consideration the preliminary results, a second set of experiments challenging the immunosorbents with non-purified HBsAg particles was performed in five purification cycles. It is well known that the most important parameter to be analysed in IAC is the selectivity, because it is a direct measure of the interaction between the target molecule with the column ligand. However, the critical parameters measured in this second set of experiments were only the adsorbed and eluted amounts of HBsAg particles and the ligand leakage. Therefore, a third set of experiments was needed to evaluate the efficiency of the CDI-monolithic immunosorbent in assuring effective HBsAg particle purification by working with the best CDI-immunosorbent variant of the second set of experiments throughout 20 purification cycles.

In general, the HBsAg particle adsorption amount was higher in CDI-CB.Hep-1 mAb-immunosorbents at: 1.0 mg/mL (3059.31±865.71 µg/mL, $n=2$), showing statistical differences in 0.5 mg/mL (2972.34±834.98 µg/mL, $n=2$) and 7.0 mg/mL (2041.40±390.20 µg/mL, $n=1$) samples, $p=0.005$. Statistical analysis also demonstrated significant differences among results in the 1.0 and 0.5 mg/mL CDI-CB.Hep-1 immunosorbents ($p=0.004$) (see **Table 2,** following page). It is interesting to note that these results were the opposite of those observed in the immunosorbent assessment done applying purified HBsAg particles, where the 7.0 mg/mL immunosorbent showed the highest elution amount. On the other hand, results of all CDI-CB.Hep-1 mAb immunosorbents evidenced the highest HBsAg adsorption capacity and amount of HBsAg particle adsorbed in the first purification cycle (**Table 1**).

Similar behaviour was observed when the eluted amount of HBsAg particles was analyzed. As in the experiment with purified HBsAg particles, the immunosorbent with the lowest eluted antigen amount was 7.0 mg/mL (628.20±91.90 µg/mL, $n=1$), while 1.0 mg/mL showed the highest antigen eluted amount (2884.50±541.00 µg/mL, $n=2$). The statistical analysis of this parameter revealed significant differences among three sets of immunosorbent results ($p=0.0002$), but no significant differences between 1.0 and 0.5 mg/mL ($p=0.1121$). This finding also confirmed the hypothesis that the antigen elution capacity and eluted amount are inversely proportional to the ligand concentration in immunosorbent-based CDI-monolithic supports (**Tables 1 and 2**).

IgG leakage was also measured to evaluate the

TABLE 2. Results of the CDI-CB.Hep-1 mAb monolithic support immunosorbents in five purification cycles applying non-purified HBsAg particles in 20 mM Tris/3 mM EDTA/0.5 M NaCl, pH 7.4, and eluted with 20 mM Tris/3 mM EDTA/0.5 M NaCl/3 M KSCN, pH 7.4. These data were extrapolated from the results obtained in CIMac monolithic analytical columns of 106 µL in volume.

Purification Cycle	Ligand Concentration (mg mAb/mL)	Antigen Adsorption Capacity (µg HBsAg/mg mAb)	Antigen Adsorbed Amount (µg HBsAg/mL)	Antigen Elution Capacity (µg HBsAg/ mg mAb)	Antigen Elution Amount (µg HBsAg/mL)	Ligand Leakage (ng IgG/µg HBsAg) (approved limit = 3.0)
1	0.5	9696.0	4848.0	8100.0	4050.0	6.7
2	—	3660.8	1830.4	3240.0	1620.0	ND
3	—	6008.0	3004.0	2934.0	1467.0	0.6
4	—	3872.0	1936.0	2214.0	1107.0	ND
5	—	5300.0	2650.0	2340.0	1170.0	2.3
Average	—	5707.2	2853.6	3756.6	1882.0	3.2
SD	—	2435.5	1217.6	2459.4	1229.7	3.1
1	0.5	6644.0	3322.0	3696.0	1848.0	5.1
2	—	6462.0	3231.0	4142.0	2071.0	ND
3	—	6092.0	3046.0	4644.0	2332.0	0.3
4	—	6560.0	2734.0	6300.0	3150.0	ND
5	—	5468.0	3122.0	3978.0	1989.0	1.7
Average	—	5777.8	3122.6	5004.0	2276.0	2.3
SD	—	1035.9	241.4	1309.5	518.0	2.4
p=	—	—	0.679	—	0.527	—
1	1.0	2930.0	2930.0	3840.0	3840.0	1.3
2	—	2174.0	2174.0	2770.0	2770.0	ND
3	—	2396.8	2396.8	2098.0	2098.0	1.8
4	—	2374.6	2374.6	2370.0	2370.0	ND
5	—	1921.3	1921.3	2277.0	2277.0	0.4
Average	—	2359.4	2359.4	2509.4	2509.4	1.4
SD	—	371.9	371.9	386.9	386.9	0.3
1	1.0	4496.8	4496.8	3430.0	3430.0	0.8
2	—	4162.0	4162.0	3000.0	3000.0	ND
3	—	3258.4	3258.4	3260.4	3260.4	1.1
4	—	3184.0	3184.0	2940.0	2940.0	ND
5	—	3695.2	3695.2	2860.0	2860.0	1.2
Average	—	3759.3	3759.3	3087.3	3087.3	1.0
SD	—	568.3	568.3	230.5	230.5	0.2
p=	—	—	0.002	—	0.232	—
1	7.0	347.4	2431.8	85.7	599.9	ND
2	—	284.9	1994.3	108.6	760.2	ND
3	—	350.4	2452.8	81.6	571.2	ND
4	—	240.6	1684.2	97.1	679.7	ND
5	—	234.9	1644.3	75.7	529.9	ND
Average	—	291.6	2041.4	89.7	628.2	—
SD	—	55.8	390.2	13.1	91.9	—
p=	—	—	0.005	—	0.005	—

LEGEND: Standard deviation (SD); Not determined because of the cost of analysis (ND)

performance of CDI-CB.Hep-1 mAb-immunosorbents in the second set of experiments. Results showed that almost all IgG average values (1.4, 2.3, and 3.2 ng IgG/µg HBsAg) were below the approved limit for the BrCN-Sepharose CL-4B immunosorbents used in the hepatitis B vaccine manufacture (≤3 ng mAb/µg HBsAg). However, CDI-CB.Hep-1 mAb-immunosorbents (0.5 mg/mL) showed an IgG leakage above the limit in the first run (5.1 and 6.7 ng mAb/µg HBsAg). There is no clear explanation for this relatively high ligand leakage from the first run of 0.5 mg/mL CDI-CB.Hep-1 mAb immunosorbents. As a matter of fact, it was an unexpected result because the ratio between CDI groups and mAb molecules should favor a higher multipoint attachment in the lowest ligand concentration supports and thus, less mAb should be released from the column. A correlation between the level of antigen elution amount and mAb released was not demonstrated either. Another interesting observation is that ligand leakage was higher when non-purified samples of HBsAg particles were applied to the columns, demonstrating the influence of the applied materials on the ligand leakage. Perhaps some kind of degradation of mAb molecule by proteases was produced under these conditions (**Table 2**).

An important factor in protein purification by immunoaffinity chromatography is to know the stability of the chromatography support applying non-purified samples and using specific sanitization conditions. It also could work in the indirect evaluation of the target and ligand protein stability, because proteins may be susceptible to inactivation or degradation owing to the physicochemical conditions employed during purification, proteolytic degradation by enzymes that may be inadvertently activated during purification, and the harsh conditions that may be used for elution of the bound material. It is well known that there are various tests that can be carried out to evaluate the performance of the supports. But it is impossible to reconcile all ideal features, which is why a compromise needs to be made. Therefore, authors decided that the most important criteria in this third study was similarity among chromatograms, antigen purity, antigen molecular homogeneity, and again, ligand leakage.

To corroborate the efficiency of the CDI-immunosorbent in more than five purification cycles, a CDI-CB.Hep-1 mAb immunosorbent at 1.0 mg/mL was challenged with non-purified HBsAg particles in 20 purification cycles. This experiment was done only with this immunosorbent because the ligand concentration determination was more reliable in 1.0 mg/mL than in 0.5 mg/mL. The antigen elution amount was the highest (2509.4±386.9 µg HBsAg/mL, cycle 1; and 3087.3±230.5 µg HBsAg/mL, cycle 2) and the ligand leakage was the lowest detected (1.4±0.3 ng IgG/µg HBsAg, cycle 1; and 1.0±0.2 ng IgG/µg HBsAg, cycle 2) in the second set of chromatography experiments (**Table 2**).

Figure 1A (following page) illustrates the high similarity between chromatograms of purification cycles 1 and 20 demonstrating indirectly the consistency in the purification of the HBsAg particles of this chromatography. Thus, it should be expected that all purification steps (adsorption, washing, elution, and sanitization) occur through the same mechanism or conditions. With regard to the eluted antigen purity, IAC is probably the most unique technique for protein purification because of appropriate purity levels achieved in one single-purification step. In that sense, purity of HBsAg particles measured by SDS-PAGE and SE-HPLC was >98%. As can be noted, the SDS-PAGE (under reducing conditions) profile was characterized by the presence of monomers, dimers, and trimers of the HBsAg particles (**Figure 1B,** following page), while the SE-HPLC results also corroborated a high molecular homogeneity of the purified HBsAg particles. These results coincided with the HBsAg particle molecular homogeneity purified by the traditional HBsAg particle purification process, which involves the application of multiple purifications steps (**Figure 1C,** following page).[7, 8]

The IgG leakage was another parameter measured to evaluate the performance of CDI-CB.Hep-1 mAb immunosorbents (1.0 mg mAb/mL). The IgG leakage was analyzed using the same non-competitive sandwich ELISA assay developed to analyze small amounts of mouse IgG in the presence of relatively high quantities of HBsAg particles.[26] Results in **Table 3** show that mAb values were below the approved limit for the BrCN-Sepharose CL-4B immunosorbents used in the hepatitis B vaccine manufacturing (≤3 ng IgG/µg HBsAg). Therefore, these results help to conclude that the 1.0 mg/mL CDI-CB.Hep-1 mAb immunosorbent kept the HBsAg particle purification capacity and ligand leakage for at least 20 purification cycles.

Finally, a parallel preliminary characterization of carbohydrate, lipid, and yeast DNA removal capacity was performed in the experiments using the non-purified HBsAg particles as applied materials. In those experiments, as is the case with natural polymers such as agarose, cellulose, or dextran, the material showed a very low non-specific carbohydrate and lipid adsorption. Conversely, an unspecific interaction between yeast DNA and monolithic

TABLE 3. Results of the ligand leakage detected in 20 purification cycles.

Purification Cycle	ng mAb/µg HBsAg
1	0.40
3	0.30
5	1.00
8	0.80
11	0.40
14	0.60
17	0.70
20	0.60
Average	0.60
SD	0.23

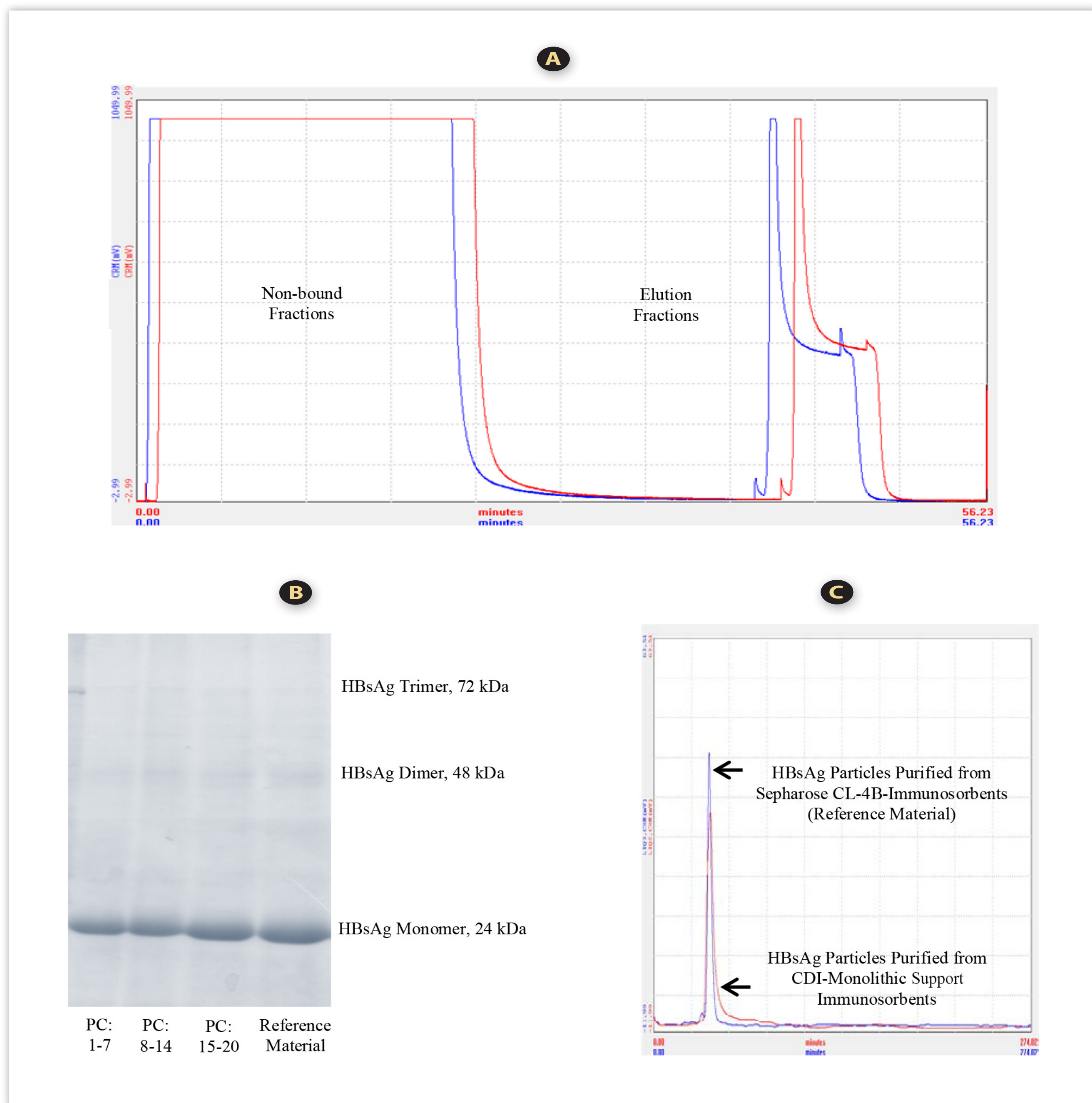

FIGURE 1. (A) Chromatograms of the HBsAg particle purification by CDI-CB.Hep-1 mAb monolithic support immunosorbents applying non-purified HBsAg particles in 20 mM Tris/3 mM EDTA/0.5 M NaCl, pH 7.4, and eluted with 20 mM Tris/3 mM EDTA/0.5 M NaCl/3 M KSCN, pH 7.4. **Blue:** Purification cycle 1; and **Red:** Purification cycle 20.

(B) Results of SDS-PAGE purity of HBsAg particles purified from CDI-CB.Hep-1 mAb monolithic immunosorbents applying non-purified HBsAg particles. **PC 1-7:** Mixture of elution samples from 1 to 7 purification cycles; **PC 8-14:** Mixture of elution samples from 8–14 purification cycles; **PC 15-20:** Mixture of elution samples from 15–20 purification cycles. **Reference material:** HBsAg particles purified from a BrCN-Sepharose CL-4B immunosorbent.

(C) Results of the molecular homogeneity of HBsAg particles purified from CDI-CB.Hep-1 mAb monolithic immunosorbents measured by SE-HPLC applying non-purified HBsAg particles. **Blue:** Reference material (HBsAg particle purified from a BrCN-Sepharose CL-4B immunosorbent); and **Red:** Purification cycle 20.

support was detected. Although these results cannot be considered conclusive because of the small number of samples assessed, special attention should be taken into account regarding the poor yeast DNA removal capacity shown by these CDI-CB.Hep-1 mAb immunosorbents to the further scale-up of the process. If it were corroborated in subsequent experiments, then an adsorption buffer different than 20 mM Tris/3 mM EDTA/0.5 M NaCl, pH 7.0, could be the solution to reducing the interaction between yeast DNA and the CDI-CB.Hep-1 mAb monolithic support, or else the washing conditions used before the elution step need to be changed as well. If the new chromatography conditions were not able to solve the unspecific interaction between yeast DNA and CDI-monolithic supports, then another solution could be the introduction of an IEC step based also on the monolithic support before the IAC. This possible solution makes sense because the IECs based on monolithic supports have large interchange capacity at very high flow-rates.[27-29] Therefore, the productivity of the whole downstream process is not affected.

Conclusions

The highest values of HBsAg particle adsorbed and eluted amounts were observed in samples of CDI-monolithic support immunosorbents at a ligand concentration equal to 1.0 mg/mL. The CDI-CB.Hep-1 mAb monolithic support immunosorbents at a ligand concentration equal to 7.0 mg/mL showed a drastic decrease in the antigen eluted amount. The SDS-PAGE purity of HBsAg particles purified from CDI-CB.Hep-1 mAb monolithic support immunosorbents was similar to the purity of HBsAg particles purified from Sepharose CL-4B immunosorbents using a reference material (≥95%). The SE-HPLC profile observed in the purified HBsAg particles purified from CDI-monolithic support immunosorbents was similar to the profile of HBsAg particles purified from the reference Sepharose CL-4B immunosorbents (only one fraction and purity ≥98%). The CDI-CB.Hep-1 mAb monolithic support immunosorbent (ligand concentration: 1.0 mg/mL) kept the HBsAg particle purification capacity for at least 20 purification cycles. The IgG content measured in samples of HBsAg particles purified from CDI-monolithic support immunosorbents during 20 purification cycles was five times lower than the limit approved by the vaccine active pharmaceutical ingredient. Therefore, monolithic support immunosorbents could be suitable for large-scale HBsAg particle purification for human vaccinations at very low mAb density and higher flow rates.

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Conflict of Interest

None of the authors have a conflict of interest with the publication of these results.

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