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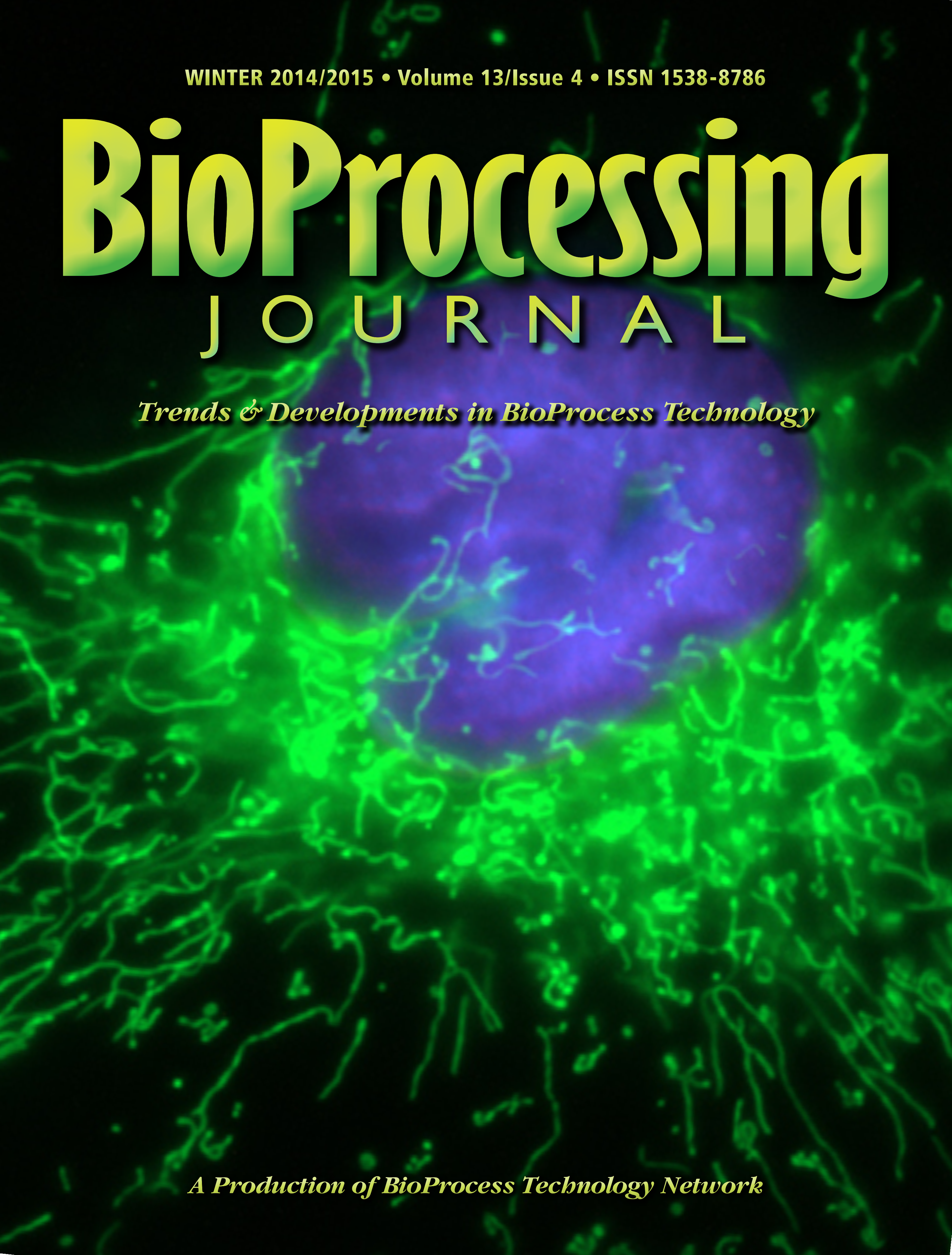

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TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

Characterization of a Lyophilized Immunoaffinity Chromatography Matrix Employed to Purify Hepatitis B Surface Antigen for Pharmaceutical Use

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Abstract

Immunoaffinity chromatography is an indispensable purification tool. However, its use has been limited by cost, purification cycle numbers, and storage requirements. Therefore, authors speculated that a possible solution to these problems could be CB.Hep-1 monoclonal antibody (mAb)-immunosorbent lyophilization. This study sought to assess the impact of the CB.Hep-1 mAb quantification by enzyme-linked immunoadsorbent assay and the CB.Hep-1 mAb-immunosorbent lyophilization process for its impact on hepatitis B virus surface antigen purification for pharmaceutical use. Study results found that CB.Hep-1 mAb lyophilization did not affect mAb purity and antigen recognition capacity. CB.Hep-1 mAb-immunosorbent lyophilization did not modify volume-weight factor, infrared spectrum, particle-size distribution, particle density and viscosity, antigen adsorption capacity, antigen elution capacity, antigen recovery, antigen purity, gamma immunoglobulin (IgG) leakage, and purification cycle number. Therefore, the lyophilized CB.Hep-1 mAb and CB.Hep-1 mAb-immunosorbents can be successfully used for hepatitis B vaccine production.

Introduction

The hepatitis B vaccine manufacturing process includes a well-characterized immunosorbent production method, which employs CB.Hep-1 monoclonal antibody (mAb) as immunoligand of the immunoaffinity chromatography matrix. This is considered the hepatitis B surface antigen (HBsAg) purification process main step.[1] However, despite CB.Hep-1 mAb-immunosorbent (liquid formulation) consistency and demonstrated effectiveness[2], several problems with processing of large volumes, stability, and transportation costs of immunosorbents make this technique relatively unattractive for industrial scale production and mainly for storage and distribution.

Therefore, authors speculated that a possible solution to these problems could be CB.Hep-1 mAb-immunosorbent lyophilization, providing a novel way of approaching the problem of CB.Hep-1 mAb immunosorbent liquid formulation. In addition, it could also be used as a model for other immunoadsorbent productions.

In regard to the lyophilization process, it is a commonly used technique to increase stability and preservation of different pharmaceutical products.[3,4] This is a drying technique that runs at low pressure and temperature, which is useful if you want to achieve a long-term stability of compounds that are not stable in solution or thermolabile. In the past fifty years, with the development achieved by pharmaceutical and biotechnological industries, the use of this conservation technique has gained prominence, because products are obtained with residual moisture values extremely low (< 5%), which does improve physicochemical and biological activity stability.[5-8]

Despite this fact, mAb and immunosorbent lyophilization has not been widely documented yet. Perhaps some damages related to physical distortion, impairment of protein secondary, tertiary or quaternary structures, and aggregation processes could provoke loss of antigen recognition capacity of mAb.[9-12]

Therefore, this study sought assessing, for the first time, efficiency of a lyophilized mAb and immunoaffinity chromatography matrix employed to purify HBsAg for pharmaceutical use.

Materials and Methods

Reagent Source

All chemical reagents used were supplied by Merck Millipore except Cyanogen bromide (CNBr), which was supplied by Sigma-Aldrich.

CB.Hep-1 mAb Source

CB.Hep-1 mAb obtained by hybridoma cell line 48/1/5/4 was generated by Fontirrochi *et al*. in 1993.[13] It recognizes the "a" determinant of HBsAg.[14] It was purified by means of protein A Sepharose® (GE Healthcare Life Sciences) affinity chromatography reaching a high purity level >95%, determined by sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE)[15] and size-exclusion high-performance liquid chromatography (SEC-HPLC).

CB.Hep-1 mAb Purity Measured by SDS-PAGE and SEC-HPLC

CB.Hep-1 mAb identity pattern and purity were determined following the procedure described by Laemmli.[15] Separated proteins were stained with Coomassie® brilliant blue R-250 (Bio-Rad Laboratories). Purity was estimated by a densitometry analysis using the GS-700 imaging densitometer with Molecular Analyst™ software (Bio-Rad Laboratories). Molecular homogeneity and purity of mAb were also estimated by SEC-HPLC using a TSKgel G3000PW (600 mm/57.5 mm ID, TosoHaas) column. Chromatographic mobile phase was 150 mM phosphate buffered saline solution (PBS) (pH 7.0), and 100 µg of sample dissolved in 150 mM PBS (pH 7.0) were directly applied into the system. Volumetric flow rate employed was 200 µL min^{-1} and absorbance was measured in an Ultrospec 2000 UV/visible spectrophotometer (GE Healthcare/Pharmacia) at 226 nm.

CB.Hep-1 mAb Quantification by Enzyme-Linked Immunoadsorbent Assay

MaxiSorp microtiter plates (Thermo Scientific) were coated with 10 µg mL^{-1} of recombinant HBsAg (rec-HBsAg) in carbonate/bicarbonate buffer (pH 9.6). After incubation, plates were washed five times. Standard and control samples were diluted in 150 mM PBS/0.2% bovine serum albumin/0.005% Tween 20 (pH 7.2) and incubated again for 20 minutes at 50 °C. Subsequently, wells were washed five times and incubated with 100 µL well^{-1} of a conjugate of goat anti-mouse polyclonal antibody preparation and horseradish peroxidase (Sigma-Aldrich) for 20 minutes at 50 °C. Plates were finally washed and reaction was revealed using *o*-Phenylenediamine dihydrochloride (OPD) as substrate and 0.015% H_2O_2 in citrate buffer (pH 5.0). The reaction was stopped by adding 50 µL of 2 M H_2SO_4 and immediately measured at 492 nm using a Thermo Scientific/Labsystems Multiskan ELISA reader.

Total Protein Quantification

Protein quantification was performed following the procedure described by Lowry *et al*.[16] Absorbance was measured at 730 nm using the Ultrospec 2000 UV/Visible spectrophotometer.

CB.Hep-1 mAb Lyophilization

CB.Hep-1 mAb (3 mL, (7.5 mg mL^{-1}) was resuspended in 150 mM PBS (pH 7.6) and dispensed prior to lyophilization into 6R vials at 3 mL per unit. A freeze-drying cycle was begun (at shelf temperature) in an SMH 15 lyophilizer (Usifroid). The temperature of the samples was decreased to −50 °C in the span of one hour. This value was maintained for five hours. The first drying was conducted at 5 °C and at a chamber pressure of approximately 150 mTorr. Chamber pressure value was maintained by nitrogen-controlled injection and the total time of this stage was 12 hours. After primary drying, shelf temperature was increased up to 25 °C and maintained for ten hours. The product in the vials was plugged under vacuum.

CB.Hep-1 mAb-Immunosorbent Preparation

CB.Hep-1 mAb immobilization was developed according to the procedure described by Hernández *et al*.[17] Coupling efficiency (ξ) was determined by an indirect method, following the formula:

$$\xi\ (\%) = \varphi/\alpha \times 100$$

Where φ is the coupled protein amount measured as the difference between initial amount of ligand, and (α) the amount detected in filtration and washing fractions after the coupling reaction.

Reactive group excess was blocked adding 100 mM glycine (pH 8.0). Immunoadsorbents were washed five times, alternately with 100 mM sodium acetate/500 mM NaCl (pH 4.0), and 100 mM $NaHCO_3$/500 mM NaCl (pH 8.3). Finally, immunoadsorbents were stored in 150 mM PBS containing 0.01% thimerosal (pH 7.2) at 4 °C.[18]

CB.Hep-1 mAb Immobilization Process Failure Mode Effects Analysis (FMEA) Application

FMEA method[19-21] application was initiated with the analysis of types and occurrence of failures in the CB.Hep-1 mAb immobilization process. For that, ten-point scales of severity (S), occurrence (O), and detection probability (P) of failures were defined for determining the risk priority number (RPN). The impact of RPN was measured as RPN = S×O×P. A scale of 1–2 was used to indicate low risk severity, low occurrence probability, and very high detection capacity. A scale of 3–5 would indicate minor inconvenience, moderate occurrence probability, and high detection capacity. A scale of 6–8 would indicate major inconvenience, high occurrence probability, and low detection capacity. A scale of 9–10 would indicate a major effect (very high severity) with a very high occurrence probability, which is practically impossible to detect. These scales were finally used for determining if a given failure was of minor importance or of high occurrence frequency. For such purposes, the following scales were also defined: RPN (< 42), risks without impact on immobilization process and do not require validation; RPN (42–143), opportunities for improvement; RPN (144–279), risks that do not affect directly immunoadsorbent quality, but if frequency of these risks increases, they could affect immunoadsorbent quality; and RPN (>279), involving risks with direct impacts on immunoadsorbent quality.

CB.Hep-1 mAb-Immunosorbent Lyophilization

CB.Hep-1 mAb-immunosorbent was resuspended in 150 mM PBS (pH 7.6) and dispensed prior to lyophilization into 6R vials at 3 mL per unit. A freeze-drying cycle began (at shelf temperature) in the Usifroid SMH 15 lyophilizer. The temperature of the samples was changed to −50 °C in the span of one hour. This value was maintained for five hours. The first drying was conducted at 5°C and at a chamber pressure of approximately 150 mTorr. Chamber pressure value was maintained by nitrogen-controlled injection and the total time of this stage was 12 hours. After primary drying, shelf temperature was increased up to 25°C and maintained for ten hours. The product in the vials was plugged under vacuum.

CB.Hep-1 mAb-Immunosorbent Volume-Weight Factor ($F_{v/w}$) Determination

CB.Hep-1 mAb-immunosorbent (1 g) was mixed with 50 mL of 150 mM PBS (pH 7.2) in a 50 mL centrifuge tube (Corning). Suspension was centrifuged (Hettich Universal 16 D-78532) at 45 ×g for three minutes.[18] The volume/weight factor was calculated as follows:

$$F_{v/w} = v/w$$

Where $F_{v/w}$: (mL g^{-1} of matrix); v: volume of immunoadsorbent after centrifugation (mL); w: weight of immunoadsorbent (g).

Sepharose CL-4B, CNBr-Sepharose CL-4B, and CB.Hep-1 mAb-Immunosorbent Infrared Spectrum Determination

A suitable amount of lyophilized Sepharose CL-4B, CNBr-Sepharose CL-4B, and CB.Hep-1 mAb-immunosorbent samples were transferred, respectively to a cuvette. A few drops of distilled water were added to allow materials to swell for 10–15 minutes. Then, samples were dried in vacuum at 40 °C for 20 minutes. In case of CNBr-Sepharose CL-4B and CB.Hep-1 mAb-immunosorbent, wet samples were washed with distilled water (10× sample volume) to remove salt, and then the excess water was removed by suction. Next, samples were dried in vacuum at 40 °C for 20 minutes. The equipment used was a Fourier transform infrared spectroscopy (FTIR) Jasco 4100 spectrophotometer with a horizontal attenuated total reflection (HATR) accessory.

Sepharose CL-4B and CB.Hep-1 mAb-Immunosorbent Density Determination

Sample density was estimated by the specific gravity method[22], which estimates the relationship between sample and distilled water density at the same temperature. Pycnometers of 5 and 10 mL were used in this determination. In addition, a Sartorius CPA224S analytical balance was used for weight determination.

Sepharose CL-4B and CB.Hep-1 mAb-Immunosorbent Viscosity Determination

Rheological determination of Sepharose CL-4B, CB.Hep-1 mAb-immunosorbent, and lyophilized CB.Hep-1 mAb-immunosorbent samples were performed using a Brookfield digital rheometer model DV3. To this end, 0.5 mL of sample was used in each measurement. Two grams of wet sample were resuspended in 4 mL of glycerol (85%) in a 50 mL Corning tube (Sigma-Aldrich).

Sepharose CL-4B and CB.Hep-1 mAb-Immunosorbent Particle-Size Distribution Determination by Dynamic Light-Scattering (DLS)

The DLS of samples performed for determining particle-size distribution was measured using a Beckman Coulter DelsaNanoC instrument. The device features a diode laser (30 mW, λ = 658 nm) capable of measuring scattered light at scattering angles of 15° and 165°, respectively. Determinations were carried out at a backscattering angle of 165°. Scattered light was detected using a photo multiplier tube and analyzed with a digital correlator. A sample volume of 1 mL in Sarstedt fluorescence cuvettes (polystyrene, [hydrodynamic diameter] hd = 1 cm) was used. For assessing correlation function g^2, properties of glycerol at 25%, refractive indices, and viscosity were measured at 25 °C (per Beckman Coulter instrument software). The cumulant method was used to calculate the z-average of (hd) and polydispersity index (PI). To perform this analysis, 2 g of matrix were always resuspended in 4 mL of glycerol.

Sepharose CL-4B and CB.Hep-1 mAb-Immunosorbent Particle Size Analysis by Microscopy

Matrix (2 g) was resuspended in 4 mL glycerol at 25% and 500 µL was used for microscopic observation (Zeiss Stemi

2000C stereo microscope) using different magnifications of 1.9, 2.4, 3.0, and 4.1. Particle size was analyzed in the Canon Utilities ZoomBrowser EX (v. 5.0.0.142) and Image J software (v. 1.45s).

Recombinant HBsAg Source

The rec-HBsAg was produced by fermentation of a recombinant strain of *Pichia pastoris* in a saline medium supplemented with glycerol, whose expression was induced with methanol.[1] In summary, cells were harvested by centrifugation and disrupted on a KDL type bed mill (Willy A. Bachofen). The disruption buffer contained 20 mM Tris-HCl/3.0 mM EDTA/300 mM NaCl/3.0 M anhydrous potassium thiocyanate (KSCN)/10 gL^{-1} sucrose (pH 8.0). The homogenate underwent acid precipitation by adding 1 M HCl (pH 4.0) and was centrifuged at 10000×g for 30 minutes. Supernatant was placed in contact with a Hyflo® Super Cel® (a flux-calcined grade of celite filter aid) equilibrated to the same pH under continuous stirring. Adsorption was allowed to take place and the filter aid was separated by centrifugation. After matrix washing with two Hyflo Super Cel volumes of 2 M KSCN solution, antigen was eluted with 20 mM Tris-HCl/3 mM/EDTA/100 gL^{-1} sucrose (pH 8.2). The rec-HBsAg was purified by a series of well-established steps including successive purification through immunoaffinity, ion exchange, and size exclusion chromatography procedures.

Immunoaffinity Chromatography Procedure

This chromatography was performed in PD-10 disposable desalting columns (GE Life Sciences) packed with 9 mL of CB.Hep-1 mAb-immunosorbents washed with 20 mM Tris/3.0 mM EDTA/1 M NaCl (pH 7.0), at 10.5 cm h^{-1} linear flow rate. Columns were directly loaded at 10.5 cm h^{-1} flow rate in saturation conditions. Washings using 20 mM Tris/3.0 mM EDTA/1.0 M NaCl (pH 7.0) were performed and the elution was carried out using 20 mM Tris/3.0 mM EDTA/1.0 M NaCl/3.0 M KSCN (pH 7.0) at a flow rate of 27 cm h^{-1}. Eluted antigen concentration was determined by UV measurement [A280 (1 cm, 1 mg mL^{-1}) = 5]. This procedure was applied in 11 purification cycles to assess stability of rec-HBsAg purification capacity of both CB.Hep-1 mAb-immunosorbents (liquid and solid formulations).

Recombinant HBsAg Purity Measured by SDS-PAGE

Samples (20 µg) were analyzed by electrophoresis on 12.5% SDS-PAGE as described by Laemmli.[15] Separated proteins were stained with Coomassie brilliant blue R-250. Purity was estimated by a densitometry analysis using the Bio-Rad imaging densitometer and Molecular Analyst software.

IgG Leakage

A sandwich ELISA determined the concentration of IgG released from the CB.Hep-1 mAb-immunosorbents. A Corning Costar® culture plate (Sigma-Aldrich) was coated with a goat anti-mouse polyclonal antibody preparation overnight at 4°C. The plate was blocked for 30 minutes at 37°C. Wells were washed, and the samples were added and incubated for three hours at 37°C with 1% non-fat milk/150 mM PBS (pH 7.2). After the plate was washed, it was incubated with 100 µL/$well^{-1}$ of the goat anti-mouse polyclonal antibody preparation and horseradish peroxidase conjugate. The reaction was then revealed using 100 µL $well^{-1}$ of 0.05% OPD and 0.015% H_2O_2 in citrate buffer (pH 5.0) and stopped with 50 µL $well^{-1}$ of 1.25 M H_2SO_4. The absorbance was measured in the Multiskan ELISA reader using a 492 nm filter.

Statistical Analysis

A one-way analysis of variance (ANOVA) test was carried out for comparing statistically experimental results. The confidence level applied was $\alpha = 0.05$. The test was applied using the software Statgraphics® Plus v. 5.0 (Statpoint Technologies) and remaining statistical analyses were done using Excel 2003 software (Microsoft).

Results and Discussion

The use of buffers and stabilizers with moderate ionic strength is enough to keep immunoadsorbent structure intact and thus, without affecting ligand recognition capacity.[17] In fact, one of these approaches has been used to store non-lyophilized CB.Hep-1 mAb-immunosorbents for up to 12 months[17,18] without modifications in the antigen recognition capacity, purified antigen purity, IgG leakage, and microbiology status. However, this storage procedure has some disadvantages, such as immunoadsorbent storage, long-term stability (> 12 months), and high probability of microbial contamination and distribution.

To overcome these constrains, we first performed a quality risk analysis[19-21] to estimate potential risks associated with CB.Hep-1 mAb-immunosorbent liquid formulation, storage, and distribution using as quality risk analysis tools, FMEA (described earlier), and the Ishikawa "fishbone" diagram. Our FMEA application results allowed us to identify eight main potential failures with 14 potential causes. From the potential causes, 14% had a direct effect on the quality of the CB.Hep-1 mAb-immunosorbent, with an NPR value equal to 400 [S (10) × O (4) × D (10)] and 200 [S (10) × O (2) × D (10)], respectively. Four (about 28%) of the potential causes were identified as improvement opportunities, and eight (57%) as potential causes that did not require validation (Table 1).

We took into consideration the fact that storage and distribution of relatively high volumes of immunoadsorbents in an H_2O-based solution is not recommended, and the RPN numbers reflect this. Our study assessed, for the first time, the impact of CB.Hep-1 mAb and CB.Hep-1 mAb-immunosorbent lyophilization processes on the CB.Hep-1 mAb-immunosorbent purification capacity of a rec-HBsAg for pharmaceutical use to minimize potential quality risks directly impacting the CB.Hep-1 mAb-immunosorbent quality.

In this sense, the lyophilization method has been employed to obtain stable formulations of mAbs.[23] However, despite the success of this method, the freeze-drying process is rarely a benign process. Damage can inactivate mAbs.[9-12] Perhaps this is one of the fundamental reasons why lyophilization of mAbs and immunosorbents has not been widely documented yet. However, in this study, purity analysis of CB.Hep-1 mAb samples showed no differences

TABLE 1. Results of quality risk analysis applied to the CB.Hep-1 mAb-immunosorbent (liquid formulation) production, storage, and distribution.

Failure Mode	Failure Effect	Potential Cause	Controls	RPN	Action
Bad immunosorbent quality	Loss of antigen recognition capacity by proteolytic degradation provoked by bacterial proteases	CB.Hep-1 mAb-immunosorbent contamination due to prolonged storage period	Immunoaffinity chromatography periodically for verifying the antigen purification capacity, release of the antibody from the immunosorbents, and to analyze the antigen recognition capacity by ELISA	400	Make process change and then validate
	No CB.Hep-1 mAb immobilization	No matrix activation with CNBr	Cyanate ester group determination	6	No validation required
		Immobilization solutions out of physico-chemical specifications	pH and conductivity checking	20	No validation required
		Failures in the stirred tank used in the immobilization	Continuous immobilization process monitoring	15	No validation required
	Low CB.Hep-1 mAb immobilization level due to cyanate ester group loss	Delay in the activation and/or immobilization reaction times	Cyanate ester group determination	10	No validation required
	Immunosorbent contamination	Errors in the material sterilization procedure	Process monitoring with indicators	27	No validation required
		Errors in the antibody sterilization procedure such as non-integrity of sterile filters used to filtrate CB.Hep-1 mAb	Sterility test	50	Identify as an opportunity for procedural improvements
		Errors in the solution sterilization procedure of the immunoadsorbent	Microbiologic control test	24	No validation required
	Poor antigen adsorption and high CB.Hep-1 mAb leakage	Inconsistencies in the quality of applied material	Non-designed control	120	Identify as an opportunity for improvement
		Bad immunosorbent packing	Theoretical plate and asymmetric factor determination	8	No validation required
		Problems with the pH of the CB.Hep-1 mAb immobilization	pH monitoring	7	No validation required
	High storage temperature of immunosorbent, which stimulates protease expression by bacteria and denaturalization of the coupled CB.Hep-1 mAb	Problems with temperature control during immunosorbent storage	Continuous temperature monitoring	80	Identify as an opportunity for improvement
	Low immunosorbent temperature, which can modify the matrix structure	Problems with temperature control during immunosobent storage	Continuous temperature monitoring	80	Identify as an opportunity for improvement
	Low stability of coupled CB.Hep-1 mAb	Problems with cold chain management and distribution	Process monitoring	200	Make changes and then validate

among lyophilized and non-lyophilized samples (Figure 1). Purity values measured by SDS-PAGE under reduction conditions were 98.5% (non-lyophilized CB.Hep-1 mAb), 98.4% (mouse IgG reference material), and 98.8% for lyophilized CB.Hep-1 mAb. While in the case of SDS-PAGE performed under non-reduction conditions, purity results were 99.6% (CB.Hep-1 mAb), 99.6% (mouse IgG reference material), and 99.0% for lyophilized CB.Hep-1 mAb. In parallel, the SEC-HPLC analysis also demonstrated the high purity and molecular homogeneity of analyzed samples: 95.9% for non-lyophilized CB.Hep-1 mAb, 95.3% for mouse IgG reference material, and 95.1% for lyophilized CB.Hep-1 mAb. Besides, the mAb specific activity calculated as the ratio between the CB.Hep-1 mAb concentration measured by ELISA and the total protein concentration measured by Lowry *et al.*[16] also corroborated the high resistance of the CB.Hep-1 mAb to the lyophilization conditions. The specific activity mean value of both mAb preparations (125 ± 0.16% (lyophilized mAb) and 111 ± 0.6% [non-lyophilized]) was close to 100%, corroborating the antigen recognition capacity and that the amount of the CB.Hep-1 mAb measured by ELISA was unmodified and thus, non-affected.

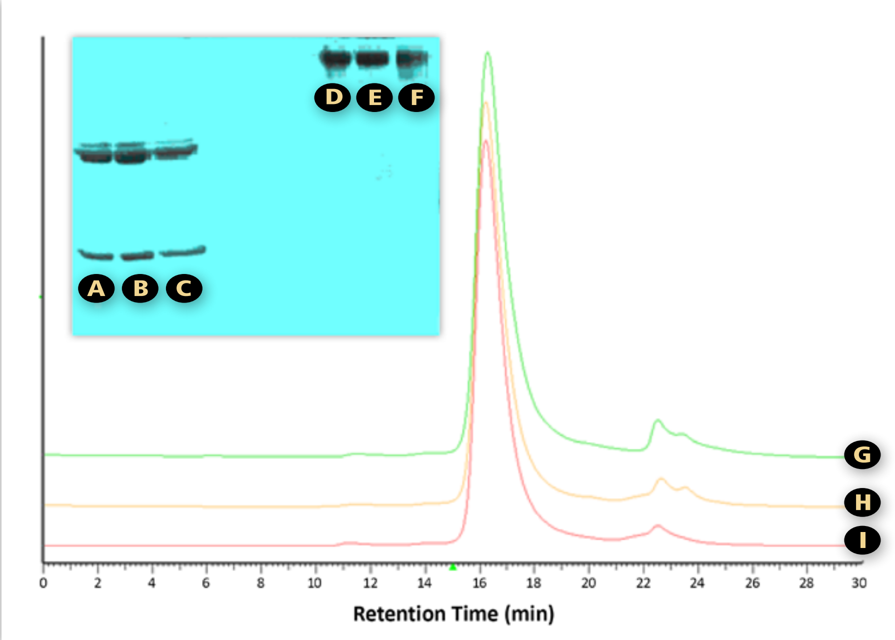

FIGURE 1. SDS-PAGE under reducing conditions: (A) non-lyophilized CB.Hep-1 mAb; **(B)** mouse IgG reference material; and **(C)** lyophilized CB.Hep-1 mAb. **SDS-PAGE under non-reducing conditions: (D)** non-lyophilized CB.Hep-1 mAb; **(E)** mouse IgG reference material; and **(F)** lyophilized CB.Hep-1 mAb. **SEC-HPLC: (G)** lyophilized CB.Hep-1 mAb; **(H)** mouse IgG reference material; and **(I)** non-lyophilized CB.Hep-1 mAb.

In order to characterize the magnitude of modifications in the Sepharose CL-4B particle structure, the $F_{v/w}$, infrared spectrum, particle density, and particle viscosity particle-size distribution were comparatively studied.

The $F_{v/w}$ analysis results showed no statistical differences between lyophilized Sepharose CL-4B (control) and lyophilized CB.Hep-1 mAb-immunosorbents. Average $F_{v/w}$ values equal to 24 ± 1 mL g^{-1} for lyophilized Sepharose CL-4B and 23 ± 1 mL g^{-1} for lyophilized CB.Hep-1 mAb-immunosorbents, $p = 0.8258$ were measured (Table 2). This parameter was considered critical for estimating, indirectly, injuries in Sepharose CL-4B particle structure and showed that the recovered

TABLE 2. Results of lyophilized Sepharose CL-4B and lyophilized CB.Hep-1 mAb-immunosorbents $F_{v/w}$ determination.

Samples	Lyophilized Sepharose CL-4B (mL g^{-1})	Lyophilized CB.Hep-1 mAb-Immunosorbent Replica 1 (mL g^{-1})	Lyophilized CB.Hep-1 mAb-Immunosorbent Replica 2 (mL g^{-1})	Lyophilized CB.Hep-1 mAb-Immunosorbent Replica 3 (mL g^{-1})
1	25	25	24	23
2	22	23	24	25
3	24	25	23	24
4	23	23	22	22
5	24	22	22	23
6	23	24	23	23
7	23	24	25	24
8	24	22	23	23
9	24	22	23	22
10	23	23	22	23
Mean	24	23	23	23
SD	1	1	1	1
P value	0.2361	0.1348	0.1518	0.1393
P value	0.8258			

volume of lyophilized CB.Hep-1 mAb-immunosorbents could be similar to that of non-lyophilized CB.Hep-1 mAb-immunosorbents. It also revealed that immunoadsorbent volume could be reduced by at least 24 times, which is very important for CB.Hep-1 mAb-immunosorbent storage, distribution, and long-term stability.

Concerning the infrared spectrum technique, it is one of the well-established experimental techniques used for polypeptide and protein structure analysis[24,25] because it is non-destructive, requires less sample preparation, and can be used under a wide variety of conditions. It is also recognized as a valuable tool for protein conformation examination in H_2O-based solutions, as well as in a dried state.[26] In general, this spectroscopy is a measurement of wavelength and intensity of infrared radiation absorption by a given sample. Polymer infrared spectral data are usually interpreted in terms of vibrations of a structural repeated unit.[27,28] The repeated units of polypeptides and proteins give rise to nine characteristic infrared absorption bands, namely, amide A, B, and I-VII. For instance, the most sensitive spectral region to protein secondary structural components is the amide I band (around 1700 cm^{-1}), which is due almost entirely to C=O stretch vibrations of peptide linkages. Frequencies of amide I band components correlated closely to each secondary structural element of proteins. The amide II band, in contrast, derives mainly from in-plane NH bending and from CN stretching vibration[29,30] showing much less protein conformational sensitivity than amide I band. Other amide vibrational bands are very complex, depending on force field details, side chain nature, and hydrogen bonding and, therefore, are of little practical use in protein conformational studies.

This assessment study of the infrared spectrum of samples was done, speculating that an indirect characterization on the influence of production steps of lyophilized CB.Hep-1 mAb-immunosorbents (matrix activation, mAb immobilization, and immunoadsorbent lyophilization) could be obtained from infrared spectrum analysis. In general, changes were observed in the position of two bands (cm^{-1}). The identified bands were at 3300, 2985, 2800, 2100, and 1756 (cm^{-1}) in Sepharose CL-4B spectrum and at 3300, 2985, 2300, 2100, and 1756 in the lyophilized CNBr-activated Sepharose CL-4B, non-lyophilized CB.Hep-1 mAb-immunosorbent, and lyophilized CB.Hep-1 mAb-immunosorbent (Figure 2). According to this, the band conversion at 2800 cm^{-1}, present in the Sepharose CL-4B in the band at 2300 cm^{-1}, was most likely due to the activation of the Sepharose CL-4B by CNBr to provoke the cyanate ester group formation.

In summary, modifications observed in the infrared spectrum have been described in the literature by various investigators, who have discovered main effects related to amide I, II, and III bands, widening of bands and disruption of bands from the rupture of hydrogen bonds, or formation of new molecular structures caused by deamidation, hydrolysis, and decarboxylation.[29,30] Besides, the lyophilized CB.Hep-1 mAb-immunosorbent infrared spectrum was almost unchanged with respect to lyophilized CNBr Sepharose CL-4B and non-lyophilized CB.Hep-1 mAb-immunosorbent infrared spectrum.

The volumetric mass density of a substance is expressed as the substance mass per unit volume. It can be used to estimate substance purity and compactness power within other applications. Therefore, in order to indirectly measure

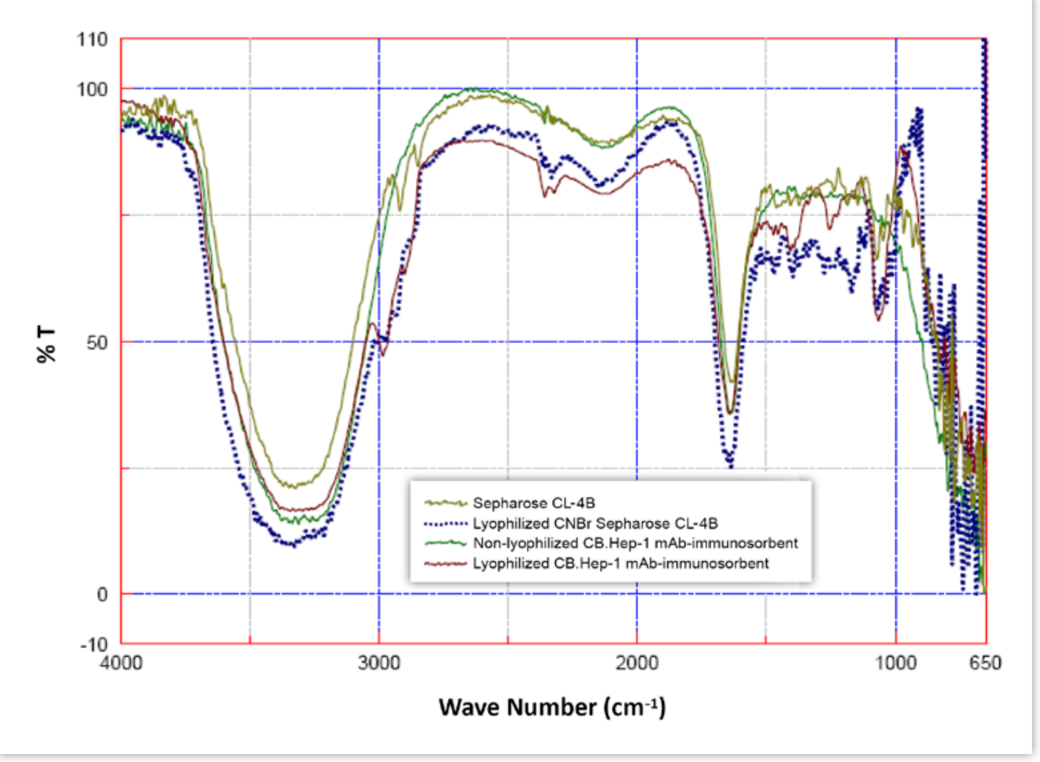

FIGURE 2. Infrared spectrum results of Sepharose CL-4B (olive green), lyophilized CNBr Sepharose CL-4B (violet), non-lyophilized CB.Hep-1 mAb-immunosorbent (green), and lyophilized CB.Hep-1 mAb-immunosorbent (dark red).

the effect of the lyophilization process on the CB.Hep-1 mAb-immunosorbent properties, the density of Sepharose CL-4B, non-lyophilized CB.Hep-1 mAb-immunosorbent, and lyophilized CB.Hep-1 mAb-immunosorbent was measured and compared under the same conditions. As can be noted from Table 3, study results demonstrated statistical differences between Sepharose CL-4B and immunosorbent density results ($p = 0.1741$). Results also showed no differences between density values of non-lyophilized CB.Hep-1 mAb-immunosorbent (1.033 ± 0.006 mg mL^{-1}) and lyophilized CB.Hep-1 mAb-immunosorbent (1.045 ± 0.011 mg mL^{-1}). Thus, differences in the densities could be attributed to the change of the matrix composition by the presence of mAb.

On the other hand, Figure 3 illustrates an example of sample viscosity determination outcomes. Results evidenced statistical differences among the slope (viscosity) of the non-lyophilized CB.Hep-1 mAb-immunosorbent (slope = 238 ± 1.19 Cp) and lyophilized CB.Hep-1 mAb-immunosorbent (slope = 231.63 ± 4.75 Cp) with Sepharose CL-4B (slope = 204.45 ± 10.38 Cp) where $p = 0.018$. However, no statistical differences were found when results of non-lyophilized CB.Hep-1 mAb-immunosorbent and lyophilized CB.Hep-1 mAb-immunosorbent were compared ($p = 0.085$). Therefore, the immunosorbent lyophilization process did not statistically modify the viscosity of immunoadsorbents. Perhaps differences observed with regard to the non-lyophilized Sepharose CL-4B can be attributed to the presence of the CB.Hep-1 mAb attached to the matrix as well.

In addition, a microscopy analysis performed for measuring particle sizes and morphology of the Sepharose CL-4B, lyophilized Sepharose CL-4B, non-lyophilized CB.Hep-1 mAb-immunosorbent, and lyophilized CB.Hep-1 mAb-immunosorbent also corroborated similarity in size and structure of beads. The bead sizes ranged as follows: Sepharose CL-4B (73.91 ± 29.86 µm); lyophilized CNBr Sepharose CL-4B (54.08 ± 27.91 µm); non-lyophilized CB.Hep-1 mAb-immunosorbent (74.64 ± 4.85 µm); and lyophilized CB.Hep-1 mAb-immunosorbent (63.95 ± 5.95 µm) (Figure 4). Besides, another analysis was

TABLE 3. Sepharose CL-4B, non-lyophilized CB.Hep-1 mAb-immunosorbent, and lyophilized CB.Hep-1 mAb-immunosorbent density analysis results.

Samples	Sepharose CL-4B (mg mL^{-1})	Non-Lyophilized CB.Hep-1 mAb-Immunosorbent (mg mL^{-1})	Lyophilized CB.Hep-1 mAb-Immunosorbent (mg mL^{-1})
Replica 1	0.985	1.023	1.036
Replica 2	0.990	1.034	1.056
Replica 3	0.986	1.039	1.042
Mean	0.985	1.033	1.045
SD	0.003	0.006	0.011
P value	0.1741		

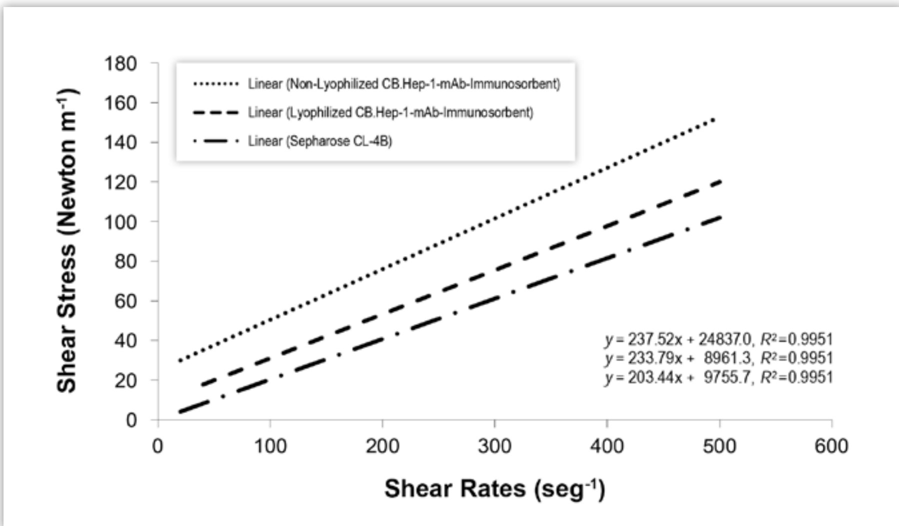

FIGURE 3. Example of viscosity determination results of Sepharose CL-4B, non-lyophilized CB.Hep-1 mAb-immunosorbent, and lyophilized CB.Hep-1 mAb-immunosorbent.

also done to corroborate the microscopy result. In this case, the particle size distribution was also analyzed by DLS. This technique is commonly used in biochemistry, biotechnology, and pharmaceutical development for measuring the size and size distribution of macromolecules and nanoparticles. In this sense, two populations of particle size were detected in the samples where one of them was not observed in the microscopy analysis of the samples. The average particle size value for the smaller particle population was 34.66 µm (Sepharose CL-4B), 31.22 µm (non-lyophilized CB.Hep-1 mAb-immunosorbent), and 38.59 µm (lyophilized CB.Hep-1 mAb-immunosorbent). The size of the larger particle size population average value was 81.53 µm (Sepharose), 78.03 µm (non-lyophilized CB.Hep-1 mAb-immunosorbent), and 69.25 µm (lyophilized CB.Hep-1 mAb-immunosorbent).

As illustrated in Figure 5, there was a small shift in both fractions of immunosorbent particle size, with respect to the Sepharose CL-4B. In this sense, it can probably be explained by the agitation effect during the mAb coupling reaction. A phenomenon with similar characteristics was observed in the report of Ioannidis.[31] Therefore, DLS results also proved the idea that lyophilization of the CB.Hep-1 mAb-immunosorbent could not modify absorbent mechanical properties such as mechanical strength, pressure, intra-particle diffusion, *etc*. These results were expected because parameters that mostly affect size and structure of the matrix are ionic strength and cooling rate, but not lyophilization.

Finally, authors proceeded to assess rec-HBsAg purification efficiency of lyophilized CB.Hep-1 mAb-immunosorbent, pretending to corroborate indirectly that the lyophilization process did not modify Sepharose CL-4B particle structure and, in consequence, CB.Hep-1 mAb-immunosorbent particle structure. As a result, no statistical differences were observed when CB.Hep-1 mAb-immunosorbent: (1) antigen adsorption capacity (71.7 ± 1.8% [non-lyophilized] and 73.0 ± 0.4% [lyophilized], $p = 0.5209$); (2) antigen elution capacity (67.5 ± 0.52%, [non-lyophilized] and 63.4 ± 2.1% [lyophilized], $p = 0.4015$); (3) total antigen recovery (52.9 ± 0.5%,

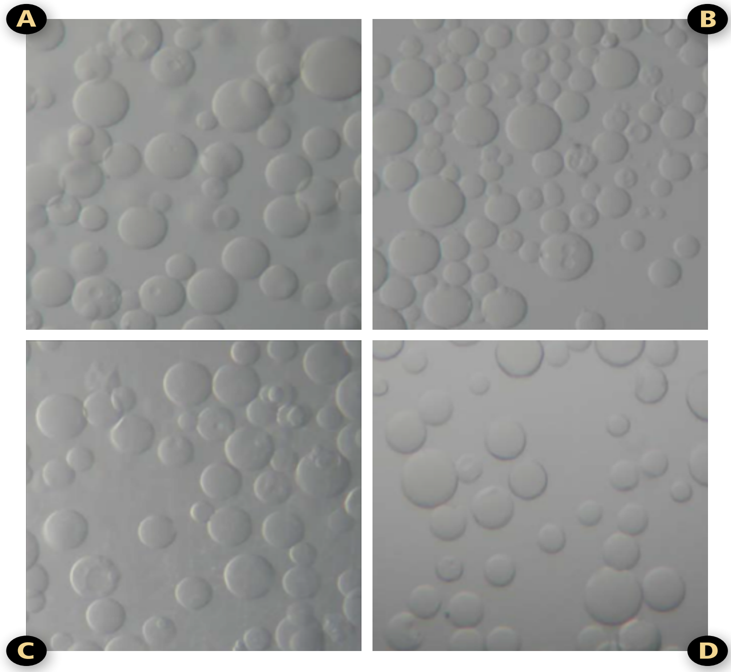

FIGURE 4. (A) Sepharose CL-4B; **(B)** lyophilized Sepharose CL-4B; **(C)** non-lyophilized CB.Hep-1 mAb-immunosorbent; and **(D)** lyophilized CB.Hep-1 mAb-immunosorbent micrographs.

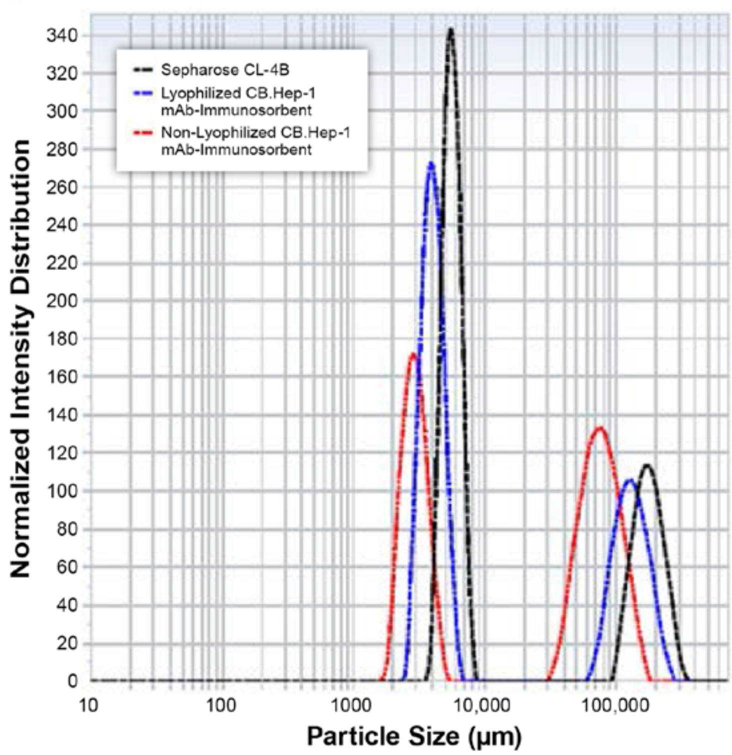

FIGURE 5. Results of Sepharose CL-4B (**black**), lyophilized CB.Hep-1 mAb-immunosorbent (blue), and non-lyophilized CB.Hep-1 mAb-immunosorbent (red) dynamic light scattering profile.

[non-lyophilized] and 48.3 ± 0.3% [lyophilized], $p = 0.1697$); (4) antigen purity (90.1 ± 0.46% [non-lyophilized CB.Hep-1 mAb-immunosorbent] and 90.2 ± 0.46% [lyophilized CB.Hep-1 mAb-immunosorbent]; and (5) immunoglobulin leakage (0.638 ± 0.217 ng mL^{-1} [non-lyophilized CB.Hep-1 mAb-immunosorbent] and 0.615 ± 0.383 ng mL^{-1} [lyophilized CB.Hep-1 mAb-immunosorbent]) were compared in 11 purification cycles ($n = 3$) (Table 4).

Therefore, the non-statistical affectation of these parameters clarified the main hypothesis of this study regarding the incapacity of the lyophilization process to affect lyophilized CB.Hep-1 mAb-immunosorbent purification efficiency. In consequence, these results open up new opportunities for storage and distribution of CB.Hep-1 mAb-immunosorbents. Authors also anticipate that results might even be improved by incorporating protective agents such as glycine, trehalose, and others that will result in greater stability of lyophilized immunoadsorbents.

TABLE 4. Results of rec-HBsAg purification using non-lyophilized and lyophilized CB.Hep-1 mAb-immunosorbents (*n*=3).

Parameters		Purification Cycle (*n*=3)											Mean	SD	*P* value (intra)	*P* value (inter)
		1	2	3	4	5	6	7	8	9	10	11				
HBsAg Adsorption Capacity (%)-1	Non-Lyophilized	82.1	73.1	83.9	66.7	69.3	74.5	77.8	77.5	63.6	66.3	75.6	73.7	6.6	0.4207	0.5209
HBsAg Adsorption Capacity (%)-2		67.9	67.1	68.6	68.4	70.3	73.7	83.0	57.1	70.7	78.2	67.4	70.2	6.6		
HBsAg Adsorption Capacity (%)-3		72.3	64.0	63.5	71.1	77.6	77.1	74.7	66.8	72.7	66.3	77.7	71.2	5.4		
HBsAg Adsorption Capacity (%)-1	Lyophilized	70.9	70.1	76.2	74.2	75.1	78.7	71.2	70.3	72.1	70.3	70.4	73.5	3.6	0.8513	
HBsAg Adsorption Capacity (%)-2		75.3	66.0	79.3	74.2	77.6	73.2	74.1	71.4	70.2	71.4	70.6	73.0	3.7		
HBsAg Adsorption Capacity (%)-3		74.1	73.1	74.7	72.3	76.3	73.8	72.4	70.6	72.6	70.2	70.2	72.7	1.9		
HBsAg Elution Capacity (%)-1	Non-Lyophilized	55.8	66.0	84.4	61.0	52.4	72.9	58.7	66.0	90.2	67.5	69.4	67.7	11.5	0.9630	0.4015
HBsAg Elution Capacity (%)-2		67.2	67.5	71.9	59.7	69.4	72.6	62.1	72.9	60.3	69.9	73.0	67.9	5.0		
HBsAg Elution Capacity (%)-3		73.0	67.5	48.1	58.8	72.6	67.4	54.3	76.3	76.3	64.9	76.2	66.9	9.6		
HBsAg Elution Capacity (%)-1	Lyophilized	68.0	74.5	65.8	67.4	62.8	57.3	69.2	67.1	56.6	58.3	54.4	63.8	6.3	0.4625	
HBsAg Elution Capacity (%)-2		75.2	90.5	69.3	65.5	66.1	55.2	67.2	63.7	56.3	54.1	55.3	65.3	10.8		
HBsAg Elution Capacity (%)-3		69.1	68.9	67.1	65.6	60.7	59.3	57.1	59.3	55.9	54.9	54.1	61.1	5.7		
HBsAg Recovery (%)-1	Non-Lyophilized	40.9	57.7	61.1	58.5	63.4	47.3	38.3	43.2	58.1	52.9	54.3	52.3	8.6	0.9513	0.1697
HBsAg Recovery (%)-2		56.2	55.7	49.8	57.6	55.0	40.2	41.6	66.3	43.6	66.8	53.0	53.2	8.9		
HBsAg Recovery (%)-3		61.8	42.2	57.9	45.4	66.7	54.0	55.5	46.1	56.9	58.2	45.6	53.4	7.7		
HBsAg Recovery (%)-1	Lyophilized	54.3	59.2	47.9	48.5	43.6	45.7	49.1	49.8	47.9	42.1	46.0	48.6	4.8	0.9597	
HBsAg Recovery (%)-2		54.3	59.7	48.5	45.5	49.8	42.5	45.1	49.8	49.5	45.1	43.2	48.5	5.1		
HBsAg Recovery (%)-3		52.3	56.3	49.3	48.3	45.3	45.0	47.9	48.2	46.3	44.3	44.1	48.0	3.9		
HBsAg Purity (%)	Non-Lyophilized	89.4	—	89.7	—	89.7	—	90.8	—	90.7	—	—	90.1	0.46	—	—
HBsAg Purity (%)	Lyophilized	89.6	—	89.8	—	90.5	—	90.5	—	90.6	—	—	90.2	0.46	—	—
Immunoglobulin Leakage (ng mL^{-1})	Non-Lyophilized	0.792	—	—	—	—	—	—	—	—	—	0.484	0.638	0.217	—	—
Immunoglobulin Leakage (ng mL^{-1})	Lyophilized	0.886	—	—	—	—	—	—	—	—	—	0.344	0.615	0.383	—	—

Conclusion

The CB.Hep-1 mAb lyophilization process did not affect mAb purity and antigen recognition capacity. The CB.Hep-1 mAb-immunosorbent lyophilization process did not modify volume-weight factor, infrared spectrum, particle density and viscosity, particle-size distribution, antigen adsorption capacity, antigen elution capacity, immunoaffinity chromatography recovery, antigen purity, immunoglobulin leakage, and purification efficiency in 11 purification cycles. Thus, lyophilized CB.Hep-1 mAb-immunosorbents can be suitable for hepatitis B vaccine production.

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