

Characterization of sodium periodate-Zetarose CL4B stability for the immunopurification of the recombinant Hepatitis B surface antigen

REPORT

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ABSTRACT

Immunoaffinity chromatography is applied for protein enrichment from heterogeneous solutions. However, some chemically-activated matrices, such as cyanogen bromide-activated Sepharose CL-4B or -Zetarose CL4B matrices, are stable for just few days. Therefore, the stability of the sodium periodate activated-Zetarose CL4B was assessed, in order to use it for the purification of the recombinant hepatitis B surface antigen (rHBsAg) for human use. The best mAb immobilization kinetic rate was measured at 2 mg/mL of mAb, reaching the maximum value at 16 h. The antigen adsorption capacity was unaffected by the activation time, the antigen-antibody application ratio, the mAb immobilization percentage, the ligand density and the ligand leakage. The dissociation constant was inversely proportional to the association constant. The minimum ligand density was lower than that of the rHBsAg large-scale purification (2,92 mg/mL de mAb), by using a cyanogen bromide-activated Zetarose CL4B purification matrix. In summary, the sodium periodate activated-Zetarose CL4B was stable for at least 100 days, proving it is suitable for the immunopurification of the rHBsAg for human vaccination.

Keywords: Agarose, hepatitis B surface antigen, immunoaffinity chromatography, monoclonal antibody

RESUMEN

Caracterización de la estabilidad de la Zetarose CL4B-activada con peryodato de sodio para la inmunopurificación del antígeno de superficie del virus de la hepatitis B recombinante. La cromatografía de inmunoafinidad se aplica para la purificación específica de proteínas a partir de soluciones heterogéneas. Sin embargo, algunas matrices activadas químicamente son estables solo unos pocos días. Por tales razones, se evaluó la estabilidad de la matriz Zetarose CL4B activada con peryodato de sodio, con vistas a emplearla para la purificación del antígeno de superficie del virus de la hepatitis B recombinante (rHBsAg) durante periodos más prolongados, con vistas a la administración del rHBsAg en seres humanos. La mejor tasa de la cinética de inmovilización del anticuerpo monoclonal (mAb) CB.Hep-1 se obtuvo a una concentración de mAb de 2 mg/mL, hasta alcanzar su valor máximo a las 16 h. La capacidad de adsorción del antígeno no se vio afectada por el tiempo de activación, la proporción de aplicación de antígeno-anticuerpo, el porcentaje de inmovilización de mAb, ni la densidad del ligando. La constante de disociación fue inversamente proporcional a la constante de asociación, y la densidad mínima de ligando fue inferior a la que se utiliza a gran escala (2,92 mg/mL de mAb). En resumen, la matriz Zetarose CL4B activada con peryodato de sodio demostró ser estable durante 100 días. Esto indica que esta matriz es adecuada para la inmunopurificación del rHBsAg para la vacunación en humanos.

Palabras clave: Agarosa, antígeno de superficie del virus de la hepatitis B, cromatografía de inmunoafinidad, anticuerpo monoclonal

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Introduction

Antibodies are fundamental proteins for antigen purification by immunoaffinity chromatography. They mediate the reversible binding interactions of the analyte of interest with the inert solid support, commonly agarose, by liquid chromatography [1].

Agarose has long been used as matrix for chromatographic separations, by displaying excellent hydrophilic properties and low nonspecific binding of main contaminants. In fact, agarose particles have an open pore structure with excellent mass transfer properties to various molecules [2], and can be activated by introducing electrophilic groups into the matrix,

making it more reactive to nucleophilic groups present in ligand or spacer molecules [3].

In this setting, several methods have been standardized to chemically activate agarose matrices. Some of them are based on introducing molecules that move ligands away from the matrix (spacer arm), thereby improving the adsorption of the target protein. There are some preferences for coupling of the ligands through a spacer arm covalently attached to the matrix [4]. However, there is also chance for coupling the ligand directly onto the matrix beads or matrices. Either the method used, the active chemical groups

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should be stable, and conditions under which the ligand is attached to the matrix must be mild enough as not to damage any components of the entire system.

Therefore, in this study, the Zetarose CL4B agarose-based matrix activated with sodium periodate was assessed to be used as support or matrix for the purification by immunoaffinity chromatography of the recombinant hepatitis B surface antigen (rHBsAg). This matrix has a particle size distribution between 45-165 μm and a 2-4 % solid material content range, with a proven effectiveness for affinity chromatography when it is activated with cyanogen bromide (BrCN) [5-6]. Noteworthy, it shows reagent safety drawbacks, such as a half-life of 3-7 days of the BrCN-activated Sepharose CL-4B or -Zetarose CL4B, forcing to evaluate new experimental conditions.

In this setting, one common method comprises the activation of aldehyde groups with sodium periodate for immunosorbent preparations [7-9].

Therefore, in this study, the Zetarose CL4B agarose-based matrix activated with sodium periodate was assessed to be used as support for the purification by immunoaffinity chromatography of the rHBsAg for human vaccination [10]. Particularly, the effects of the CB.Hep-1 mAb immobilization kinetics and the in-batch adsorption capacity (at 0, 60 and 100 days) were analyzed. Furthermore, binding parameters (adsorption capacity, association and dissociation constants, elution capacity, adsorption efficiency, elution efficiency, recovery and ligand leakage) were determined. All these could lead to an improved affinity chromatography purification process of the rHBsAg using Zetarose CL4B with the CB.Hep-1 mAb as ligand.

Materials and methods

Monoclonal antibody and rHBsAg sources

The CB.Hep-1 mAb, secreted by the hybridoma cell line CB.Hep-1, was highly purified (> 97 % purity) from ascites fluid and used as ligand to conduct the experiments [6]. The rHBsAg was produced by fermentation of a recombinant strain of *P. pastoris* (C-226) in saline medium, supplemented with glycerol. The rHBsAg expression was induced with methanol, and the samples harvested for initial purification, showing 10-15 % purity, were used for immunoaffinity chromatography [10].

Activation of Zetarose CL4B

Zetarose CL4B (Emp-BIOTECH, Buch, Germany) was first washed five times with purified H_2O at a 2:1 ratio (matrix:water), and once the matrix settled, the supernatant was completely drained. Subsequently, it was reacted with a 5 mM NaIO_4 solution (Merck, Darmstadt, Germany) at a 1:1 ratio for 5 min (Stuart see-saw rocker, Stafford shire, UK), at room temperature. Next, the supernatant was completely drained and the activated matrix washed five times with purified water (2:1, matrix:water). Then, the previously drained activated matrix was resuspended at 50 % of the final matrix volume and stored at 4 °C until use. This simple procedure was carried out in a GLP-complying laboratory (CIGB, Havana, Cuba).

Immunosorbent preparation

The sodium periodate-activated Zetarose CL4B matrix was previously washed five times with purified H_2O (2:1, matrix:water ratio). The matrix was let to settle down and the supernatant was completely drained. The Zetarose CL4B volume needed for the mAb immobilization was obtained, at a 1.3-1.5 mL/g volume/weight factor. Subsequently, the drained matrix was washed five times with 150 mM phosphate-buffered solution (PBS), pH 7.4, at a 2:1 ratio (matrix: buffer). Next, mAb molecules were coupled to the activated matrix (Stuart see-saw rocker, Stafford shire, UK); 1:1 ratio (volume of matrix: volume of mAb solution). Then, 5 mg of NaCNBH_3 per milliliter of matrix were dissolved in 150 mM PBS, pH 7.4, and added to the matrix mixture at a ratio of 5 mg NaCNBH_3 /40 μL 150 mM PBS, pH 7.4) for immobilization under gentle stirring conditions.

The supernatant was drained and collected, and the immunosorbent washed twice with 150 mM PBS, pH 7.4 (2:1 matrix: buffer) and supernatant drained again. About 5 mg NaCNBH_3 /mL Zetarose CL4B were weighed (Sartorius, Götting, Germany) and dissolved in 1 M ethanolamine (Riedel-de Haen, Seelze, Germany), pH 8.0 at a 2:1 ratio (matrix: solution). The mixture was added to the immunosorbent and stirred (Stuart see-saw rocker, Stafford shire, UK) for 3 h. The immunosorbent was completely drained and washed with 150 mM of PBS, pH 7.4, and further resuspended and stored in 150 mM PBS, pH 7.4, at 4 °C. Collected fractions were measured by UV/vis to estimate the immobilization percentage and the ligand density. All the procedures were done in a laboratory complying with Good Laboratory Practices.

Determination of CB.Hep-1 mAb and rHBsAg concentration

Highly purified samples of the CB.Hep-1 mAb recognizing the rHBsAg were diluted, to determine their absorbances at 280 nm. Next, sample absorbances were measured on a visible UV ULTROSPEC 2000 spectrophotometer (Pharmacia Biotech, England).

Measurement of CB.Hep-1 mAb leakage from immunosorbents by ELISA

Briefly, a Costar® plate (Costar, Cambridge, USA) was coated with a sheep anti-mouse polyclonal immunoglobulin and incubated at 4 °C, overnight. The plate was further blocked with 1 % non-fat milk/150 mM PBS, pH 8.0, at 37 °C for 30 min. Next, wells were washed and the samples eluted from the immunosorbents were added, and incubated at 37 °C for 1 h, with 1 % non-fat milk/150 mM PBS, pH 8.0. After a washing step, the plate was incubated with 100 μL /well of a goat anti-mouse polyclonal immunoglobulin-horseradish peroxidase conjugate (Sigma-Aldrich). Subsequently, the reaction was revealed using 100 μL /well of 0.05 % OPD 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 . Absorbance was measured at 492 nm in a Multiskan ELISA reader (Labsystems, Helsinki, Finland), using a 492 nm filter.

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Immobilization kinetics of CB.Hep-1 mAb to the sodium periodate-activated Zetarose CL4B

The CB.Hep-1 mAb was added at three initial concentrations (1.0, 1.33 and 2.0 mg/mL), three replicates each, for an expected ligand density equal to 4 mg/mL in the immunosorbents. The coupling was performed on a rocker shaker (Stuart see-saw rocker, Stafford shire, UK), at 10 osc./min, with sampling after 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, and 960 min.

Estimation of the in-batch rHBsAg maximum adsorption capacity

The stability of the sodium periodate-activated matrix was evaluated in terms of rHBsAg adsorption capacity. For this, immunosorbents were produced containing this matrix upon storage of the activated matrix for 0, 60 and 100 days. Then, rHBsAg samples were applied at 150, 160, 170, 180, 190, 200, and 220 µg rHBsAg/mg mAb. In all the cases, the samples were left into contact with the matrix for 20 h, under agitation at 10 osc/min, in a rocker shaker (Stuart rocker, Stafford shire, UK).

Calculation of the in-batch association and dissociation constants between the rHBsAg and CB.Hep-1 mAb

The association and dissociation constants of the system were calculated, using an immunosorbent at 3.97 mg/mL ligand density. The antigen samples (150, 180 and 220 µg rHBsAg/mg mAb) were incubated with 0.5 mL of the immunosorbent (0.385 g), and incubated at room temperature for adsorption, under agitation at 10 osc./min for 24 h in a rocker shaker (Stuart see-saw rocker, Stafford shire, UK). Then, the rHBsAg was eluted for 3 h under the same conditions.

Indirect determination of the stability of sodium periodate-activated Zetarose CL4B in the immunosorbents

Immunoaffinity chromatography was performed in PD-10 columns loaded with 8.85 mL of immunosorbent, equilibrated with 45 mL of 20 mM Tris/3 mM EDTA/0.5 mM NaCl, pH 7.39, at a 2.12 cm/h linear flow rate. Next, the immunosorbent was washed with 20 mM Tris/3 mM EDTA/1 mM NaCl, pH 7.38 at 3.55 cm/h linear flow rate. Subsequently, the antigen was eluted using 20 mM Tris/3 mM EDTA/1 mM NaCl/3 M KSCN, pH 7.6, operated at 3.55 cm/h linear flow rate.

Statistical analyses

Statistical analyses were performed with the aid of the Statgraphic Centurion XV software, version 15.2.06 (Statistical Graphics Corp., USA), with a 95 % significance level. Chromatographic parameters, and the batch and column capacity calculations were made using Microsoft Excel (Microsoft, USA).

Results and discussion

Several procedures used for coupling proteins to agarose-based matrices use chemical activation of agarose with BrCN [11], because it is a simple and fast method. Despite, other procedures have been developed for agarose activation, producing more stable activated matrices, and immunosorbents with

high antigen purification capacity and imperceptible ligand leakage. Among them, activation with sodium periodate is one of the most common, which relies on the oxidation of the agarose cis-proximal hydroxyl groups by sodium metaperiodate (NaIO_4), to generate functional aldehyde groups [12]. Therefore, in this work, the sodium periodate-activated Zetarose CL4B matrix was analyzed, for using it to immunopurify the rHBsAg produced in *P. pastoris* yeast intended for human vaccination, to replace the unstable BrCN-activated Sepharose CL-4B and Zetarose CL4B.

Immobilization kinetics of CB.Hep-1 mAb to sodium periodate-activated Zetarose CL4B

The CB.Hep-1 mAb immobilization kinetics was analyzed, with the concentration of the CB.Hep-1 mAb to be immobilized as the main parameter (Figure). As shown, the amount of immobilized CB.Hep-1 mAb increased (48.9-92.0 %) with the increase on its concentration (1.0-2.0 mg/mL), consistent with reports by Montanier *et al.*, 2019 [13]. Probably, the low percentage of immobilized CB.Hep-1 mAb, estimated at 1 mg/mL, could come from a kinetic effect produced at low antibody concentrations. Furthermore, there could be denaturing effects on the antibody molecule, under the relatively slow immobilization reaction conditions tested. After 16 h of exposure, some protein molecules may undergo structural changes, which could lead to aggregation and precipitation, ultimately limiting the immobilization reaction [14].

Measurement of rHBsAg adsorption capacity of sodium periodate-activated Zetarose CL4B immunosorbents in batch

The maximum adsorption capacity of the Zetarose CL4B activated with sodium periodate was measured in batch at 0, 60 and 100 d (Table 1). As can be seen, there were no significant differences between time-points (641.53 ± 100.27 , 612.47 ± 70.23 and 621.15 ± 75.42 µg rHBsAg/mg mAb on days 0, 60 and 100, respectively ($p = 0.8000$). Non-activated Zetarose CL4B was used as a negative control, which absorbed only 2 % of the total amount of rHBsAg applied. This indirectly confirmed the great stability of active sites (aldehydes) generated in the matrix during the activation process, with a remarkable positive effect on the control and consistency of the mAb immobilization process.

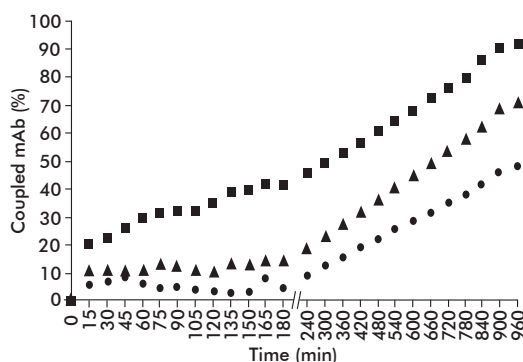


Figure. Results of CB.Hep-1 mAb immobilization kinetics on sodium periodate-activated Zetarose CL4B.

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Previous reports have demonstrated the stability of the sodium periodate-activated matrices, but just for short periods. For instance, Fernández-Lorente *et al.* [15] demonstrated the stability of highly activated glyoxyl support for seven days. Moreover, we have shown that that non-totally dried Sepharose CL-4B, Applipurose CL-4B and Zetarose CL4B activated with BrCN have a very short stability, less than one week. Therefore, current results expand the knowledge of the agarose-based matrix stability for protein immobilization, supporting its effective application for mAb immobilization.

Calculation of association and dissociation constants between rHBsAg and the immunosorbent

Affinity is the term used to describe the strength of a single bimolecular interaction between a ligand and a specific target. If the antibody is able to bind to the target through two or more binding sites, these multiple interactions can create synergies, thereby enhancing the apparent affinity. This effect is commonly called avidity. Furthermore, rHBsAg is a particle of approximately 2×10^6 Da, formed by multiple copies of an antigenic monomer [16]. Therefore, the antigenic determinant is repeated several times, mediating a strong avidity effect, which makes difficult the estimation of the association and dissociation constants between the rHBsAg and the CB.Hep-1 mAb-immunosorbents. Despite, knowing these parameters is relevant, since not always a high association constant is directly proportional to the dissociation constant, leading to a reduction in the chromatographic recovery. Hence, both parameters were calculated for the rHBsAg and the immunosorbent at different applied antigen-antibody ratios (150, 180, and 220 $\mu\text{g rHBsAg/mg mAb}$).

There was not a significant increase in the association constant with the increase of the rHBsAg application ratio ($K_a = 1.02, 1.23$ and 1.5 , for 150, 180, and 220 $\mu\text{g rHBsAg/mg mAb}$, respectively). On the contrary, the dissociation constant decreased with the increase of the rHBsAg application ratio ($K_d = 0.98, 0.82, 0.67$, respectively). These may be explained by the sustained number of mAb molecules (1.61×10^{16})

Table 1. Sodium periodate-activated Zetarose CL4B adsorption capacity measured in batch

Initial ratio ($\mu\text{g rHBsAg/mg mAb}$)	Adsorption Capacity ($\mu\text{g rHBsAg/mg mAb}$)/Adsorption Efficiency (%)								
	Time (d)	Coupled mAb (%)	Ligand density (mg/mL)	Time (d)	Coupled mAb (%)	Ligand density (mg/mL)	Time (d)	Coupled mAb (%)	Ligand density (mg/mL)
	0	99.38	3.97	60	94.51	3.78	100	98.87	3.96
150		500.96/84.12			506.28/89.29			495.70/83.45	
160		559.53/88.09			551.07/91.12			583.92/92.16	
170		588.70/87.23			583.74/90.84			600.71/89.23	
180		632.22/88.47			621.73/91.38			625.97/87.82	
190		686.05/90.95			667.14/92.89			633.48/84.19	
200		753.17/94.86			648.03/85.72			667.80/84.32	
220		770.05/88.17			709.32/85.30			740.46/84.99	
p					0.8000/0.2253				

in these immunosorbents, which was always higher than the number of applied antigen molecules ($1.18 \times 10^{14}, 2.17 \times 10^{14}$ and 2.65×10^{14}). Therefore, there was always enough mAb to interact with the applied rHBsAg, favoring the association constant and disfavoring the dissociation constant, and the resulting efficiency of the antigen elution and recovery. Perhaps, the decrease in the K_d value could be explained by the increase in the diffusion of the antigen into the gel, due to a higher antigen application ratio, favoring the interaction between the antigen and mAb molecules.

Evaluation of the sodium periodate-activated Zetarose CL4B immunosorbents in chromatography for 0, 60 and 100 days

Six parameters were measured for the immunosorbent assessment during 100 days: adsorption capacity, elution capacity, adsorption efficiency, elution efficiency, recovery and ligand leakage (Table 2). The simultaneous influence of ligand density on the efficiency of the chromatographic parameters were tested for the immunosorbents at different ligand densities (1.93, 2.92, 3.95 and 4.94 mg/mL), supporting the selection of the minimum ligand density to be used at large scale.

The analysis of the data demonstrated a great stability of the sodium periodate-activated Zetarose CL4B for 100 days, since no significant differences were detected in not any parameter at each ligand density.

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Table 2. Results of chromatographic parameters measured in the stability study of CB.Hep-1-Immunsorbents based on sodium periodate-activated Zetarose CL4B in 100 days

Ligand density	Chromatographic parameters						Ligand leakage (ng mAb/ $\mu\text{g rHBsAg}$)
	Fermentation time (d)	Adsorption capacity ($\mu\text{g rHBsAg/mg mAb}$)	Elution capacity ($\mu\text{g rHBsAg/mg mAb}$)	Adsorption efficiency (%)	Elution efficiency (%)	Recovery (%)	
1.93	0	74.20 $\pm$ 12.85	15.29 $\pm$ 2.55	47.68 $\pm$ 8.53	20.06 $\pm$ 1.47	9.83 $\pm$ 1.73	–
	60	70.29 $\pm$ 17.03	16.97 $\pm$ 13.13	44.09 $\pm$ 10.03	23.36 $\pm$ 14.17	10.58 $\pm$ 8.22	–
	100	75.24 $\pm$ 11.75	19.66 $\pm$ 5.33	47.26 $\pm$ 6.79	26.65 $\pm$ 8.13	12.35 $\pm$ 3.24	–
	p	0.9033	0.8195	0.8577	0.7095	0.8392	–
2.92	0	88.08 $\pm$ 5.29	53.14 $\pm$ 14.76	53.16 $\pm$ 4.28	59.77 $\pm$ 13.29	34.69 $\pm$ 9.62	–
	60	88.09 $\pm$ 12.49	55.93 $\pm$ 7.89	55.49 $\pm$ 7.48	63.86 $\pm$ 7.76	35.20 $\pm$ 4.24	–
	100	95.95 $\pm$ 7.06	60.70 $\pm$ 2.26	61.49 $\pm$ 4.89	63.56 $\pm$ 6.48	38.89 $\pm$ 1.53	–
	p	0.4943	0.6513	0.2623	0.8499	0.6752	–
3.95	0	97.22 $\pm$ 14.75	65.06 $\pm$ 3.79	62.88 $\pm$ 10.89	56.73 $\pm$ 9.47	35.62 $\pm$ 7.67	–
	60	96.24 $\pm$ 2.48	69.24 $\pm$ 8.24	60.67 $\pm$ 1.44	72.06 $\pm$ 10.09	43.66 $\pm$ 5.15	–
	100	96.10 $\pm$ 1.73	67.09 $\pm$ 0.84	60.78 $\pm$ 1.43	69.82 $\pm$ 0.87	42.43 $\pm$ 0.75	–
	p	0.9854	0.6449	0.8944	0.1128	0.2209	–
4.94	0	95.57 $\pm$ 2.82	70.99 $\pm$ 5.37	61.89 $\pm$ 1.95	74.40 $\pm$ 7.31	46.04 $\pm$ 4.76	1.96
	60	96.88 $\pm$ 3.36	70.79 $\pm$ 4.94	62.06 $\pm$ 0.82	73.01 $\pm$ 2.63	45.32 $\pm$ 2.13	0.17
	100	99.51 $\pm$ 5.52	70.82 $\pm$ 1.36	63.49 $\pm$ 3.77	71.37 $\pm$ 5.26	45.18 $\pm$ 0.66	0.08
	p	0.5212	0.9982	0.7093	0.7970	0.9343	–

Otherwise, significant differences were estimated among the 1.93 mg/mL ligand density value and the others. Thus, it is proposed to use the 2.92 mg/mL ligand density as the minimum at large-scale CB.Hep-1 mAb immobilization. These results contrast with those obtained by Gómez *et al.* [17], when optimizing the CB.Hep1 mAb coupling in Sepharose CL-4B previously activated by the cyanogen bromide method, which showed a minimum working ligand density of 3.2 mg/mL [17].

Conclusions

It was demonstrated that the immobilization kinetic rate of the CB.Hep-1mAb is slow, reaching a maximum value at 16 h. The antigen adsorption capacity remained unaffected by the activation time, the antigen-antibody application ratio, the mAb immobilization percentage and the ligand leakage and density. The dissociation constant was shown to be inversely proportional to the association constant. A minimum ligand density of 2.92 mg/mL is proposed to be used

in large-scale mAb immobilization. Overall, the sodium periodate-activated Zetarose CL4B was reliably stable for at least 100 days. It is very convenient for the replacement of the most popular matrix activation procedure (BrCN), which is quite instable and has many biosafety weaknesses, for vaccine production. It also allows to scale up the matrix activation procedure, since it is stable for at least 100 days.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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