Preparation, characterization and performance of chromatographic membrane with phenylboronate ligands for biomolecules purification

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Abstract
New methods of separation and purification of biological products have been investigated in the chemical, pharmaceutical and biological industry. An example of such purification methods often used is the membrane-based chromatographic separation. This type of chromatography is becoming often used for separations in bioprocesses because it allows faster mass transport through the membrane, increasing the productivity of the process. The membranes adsorbents used can be cellulose-based membranes which were subjected to several derivatization processes in order to present units of a desired functional group on their surface. These processes are series of chemical reaction steps developed at the membrane surface. The present work reports the development of a derivatization process on microporous membranes with phenyl boronic acids and later their application as potential adsorbers for the purification of two different types of biological products, namely plasmid DNA and monoclonal antibodies, by membrane chromatography. Two boronic acid derivatives have been chosen: 3-carboxyphenyl-boronic acid and 4-carboxyphenyl-boronic acids. The derivatization of the commercial cellulose-based membranes, pre-activated with aldehyde groups was based on a two-step methodology: (i) membranes were first activated with an aliphatic diamine spacer arm and (ii) were afterwards functionalized with the phenyl boronic acid ligands through carbodiimide coupling. According to the TNBS method the first step was successfully accomplished, an average density of amines on derivatized membranes of 0.012 μeq/cm² was obtained and the chromatographic experiments showed that the derivatized membranes have acquired insufficient adsorption capacity for the solutes that were loaded, rendering unfeasible for intended separations in this study.

Keywords: Membrane chromatography; Plasmid DNA (pDNA); Antibodies; Boronic acid derivatives.

1. Background and Objectives

Chemical or biochemical processes, designed to produce compounds with certain specifications, require downstream operations to separate, concentrate and purify species in different streams of such processes. The latter are fundamental to many industries in the biotechnology sector, particularly if dealing with bioactive pharmaceutical molecules. In most cases, purification steps in an existing production process are specific to a particular biomolecule and can reach about 90% of the final cost of the product. Some separation techniques require many process steps, which hinder their application on a large scale as it would be economically unviable. Therefore, it is necessary to develop innovative techniques or improve current techniques to optimize existing processes and make them economically viable without reducing the yield of the process.[1]

In this context, the membrane separation processes have emerged as very promising alternatives to classical separation processes in the
For convenience in their purification and characterization, boronic acids are often best handled as ester derivatives, in which the two hydroxyl groups are masked. Likewise, transformation of the hydroxyl groups into other substituents such as halides may also provide the increased reactivity necessary for several synthetic applications. Recently, the increasing popularity of boronic acids as synthetic intermediates has motivated the development of solid supports like membranes and linkers to allow their immobilization and facilitate purification operations or derivatization. The appeal of these methods is particularly apparent in view of the difficulties often encountered in isolating pure boronic acids from both aqueous and organic solvent systems. In this functional group, ligands derivatives of boronic acid, especially PBA, have shown great potential of application in the purification processes of several biological compounds with therapeutic activity, for example, the direct capture of antibodies (glycoproteins) from the cell culture medium [6] and the removal of RNA and lipopolysaccharides from plasmid DNA (pDNA) containing lysates.

These PBA ligands have the ability to interact with compounds bearing cis-diol groups by forming a covalent ester bond and therefore can interact with carbohydrates, glycosylated proteins (glycoproteins), ribonucleic acid (RNA), lipopolysaccharides, among others.

The goal of this work was to develop membrane absorbers derivatized with phenylboronate able to separate and purify monoclonal antibodies (mAbs) and pDNA using a membrane chromatographic technique. Therefore, various techniques have been used to link a phenylboronate group to the membrane surface. Afterwards, quantitative tests were applied to choose membranes that showed higher derivatization yield, in order words, a greater amount of ligands on their surface. Finally, the membranes already functionalized with boronic acid were tested for their ability for establishing affinity interactions between the matrix and the biological products subjected to the chromatographic techniques.

2. Materials and Methods

2.1. Preparation of disc membranes

The derivatization process of Sartobind® Aldehyde membranes with nominal pore size of 0.45 μm (Sartorius Stedim Biotech GmbH) started with the cutting of disc membranes with 25 mm diameter from a Din A4 sheet. Fourteen membrane discs were cut and stored in 25 mL vials labeled accordingly to the further use. During the process, more disc membranes were cut to be used in different repetitions of derivatization processes evaluated in this work. These disc membranes were separated into two lots: lot 1 contained derivatized membranes...
without the presence of N-hydroxsuccinimide (NHS) and lot 2 contained the derivatized membranes in the presence of NHS.

2.2. The amination procedure

The derivatization procedure started with the preparation of aminated membranes. First, the membranes were washed three times with MilliQ water, at a water to membrane area ratio of 0.5 mL/cm², to remove protective and moistening chemicals. After washing, each disc was dehydrated at 55°C for 30 min in a Universal Fan Oven (Memmert UFB500). In this first step two experiences were performed: (i) the aldehyde ligand groups reacted with 5 mL of 0.5M NaOH solution per membrane piece at 60°C for 2h (or room temperature overnight) under orbital incubation at 200-300 rpm in a Heidolph Titramax 1000 Plate Shaker; or (ii) aldehyde pre-activated membranes reacted with 5 mL of 200mM 1,6-hexanediamine (HEDA) in NaOH at pH 11 for 12 h at 45°C, under similar 200-300 rpm incubation resulting in aminated membranes. The membranes were afterwards washed with 3 volumes of MilliQ water and dried in the oven for one hour.

2.3. Carbodiimide-mediated coupling of ligands

In the second step, aminated membranes were linked to carboxylic groups in the presence of a soluble carbodiimide [8], particularly the 1-ethyl-3-[dimethylaminopropyl]carbodiimide hydrochloride (EDC), using two reaction protocols. This reaction was performed for two phenylboronate ligands: 3-carboxyphenyl-boronic acid (3-CPBA) and 4-carboxyphenyl-boronic acid (4-CPBA). For simple understanding, the reaction with 3-CPBA acid was named ligand one and the reaction with 4-CPBA was named ligand two. In protocol 1, a fresh solution containing 100 mM EDC in 50 mM phosphate buffer at pH 6.0 was prepared. To 25mL of this solution was added 3mg of 3-CPBA (EDC/3-CPBA stock solution) and to other 25mL was added equal amount of 4-CPBA (EDC/4-CPBA stock solution), because it was considered that the concentration of CPBA should be ten times higher than the concentration of amines. Then, each aminated membrane disc was submerged in 5mL of each EDC/ligand stock solution for 16h at room temperature, for reaction. Afterwards, ligand-immobilized membranes were washed three times with phosphate buffer to remove the excess reagents and were stored in a buffer solution at 4°C. Moreover, in this step, some of the aminated membranes (those of lot 2) were also linked to carboxylic groups in the presence of EDC, PBA-derivatives 3-CPBA or 4-CPBA and NHS. Again, the NHS aimed to increase the yield of the reaction of carbodiimide coupling to aminated membranes. A fresh solution (80 mL) containing 0.0035M EDC, 0.0035M NHS and 0.0007M CPBA in 0.5M phosphate buffer at pH 6.0, was prepared and incubated for 4h at room temperature. Four milligrams of 3-CPBA acid or 4-CPBA were added to 40 mL of this solution. Then each aminated membrane reacted with 3 mL the EDC/NHS/CPBA buffered solution for 7h at room temperature. Afterwards, the membranes were washed three times with 0.5M buffer solution to remove the excess reagents and maintained on the same solution at 4°C.

In the protocol 2, aminated membranes were also linked to carboxylic groups in the presence of EDC with PBA-derivatives 3-CPBA or 4-CPBA, but at different environmental conditions. A fresh solution (50 mL) containing EDC, at a concentration 10 times higher than the concentration of amine previously quantified, in 0.5M phosphate buffer at pH 8.0, was prepared and incubated for 4h at room temperature. Three milligrams of 3-CPBA acid or 4-CPBA were added to 25 mL of this solution. Then, each aminated membrane reacted with 5 mL the EDC/CPBA buffered solution for 15 h at room temperature. Afterwards, the membranes were washed three times with 0.5M buffer solution to remove the excess reagents and maintained on the same solution at 4°C. Moreover, in this step, some of the aminated membranes (those of lot 2) were also linked to carboxylic groups in the presence of EDC, PBA-derivatives 3-CPBA or 4-CPBA and NHS. Again, the NHS aimed to increase the yield of the reaction of carbodiimide coupling to aminated membranes. A fresh solution (80 mL) containing 0.0035M EDC, 0.0035M NHS and 0.0007M CPBA in 0.5M phosphate buffer at pH 8.0, was prepared and incubated for 4h at room temperature. Four milligrams of 3-CPBA acid or 4-CPBA were added to 40 mL of this solution. Then each aminated membrane reacted with 3 mL the EDC/NHS/CPBA buffered solution for 7h at room temperature. Afterwards, the membranes were washed three times with 0.5M buffer solution to remove the excess reagents and maintained on the same solution at 4°C.

2.3. Amines quantification

The reagent 2,4,6-trinitrobenzenesulfonic acid (TNBS) was used to determine the concentration of amines at the membrane surface. The TNBS method relates directly the primary amine molecules present on the membrane surface with the absorbance of the trinitrophenol molecules released from it under alkaline hydrolysis [8, 9].

In this step, after the 12 h of reaction with HEDA, disc membranes were submerged in 5 mL of 10 mM TNBS solution prepared with 100 mM PBS buffer at pH 9, for 2 h at room temperature in a Heidolph Titramax 1000 Plate Shaker, under 200-300 rpm, to produce a chromogenic derivative which was bound to the membrane. The TNBS solution is light
sensitive, therefore the reaction flasks were covered with black cloth. Afterwards, the membranes were washed with MilliQ water to remove the excess reagent, wash water was discharged and 15 mL 2 M KOH solution was added to membranes and left to react for 3h at 50°C. The addition of KOH leads to the hydrolysis of the chromogenic derivative, with 2,4,6-trinitrophenol being released.

The amine concentration and the process efficiency was determined by absorbance of the released product in the supernatant solution, measured at 358 nm in a Hitachi U-2000 Spectrophotometer against a blank sample of 2 M KOH solution.

Later, the concentration was determined by Lambert-Beer law.

2.4. Phenylboronate quantification

The phenylboronate quantification by alizarin red S (ARS) method is based on the utilization of the colorant ARS as an analytical reagent for the determination of a desired element, in this case boron. The methodology applied for analysis of phenylboronate ligand capacity relies on ARS and is performed in three steps. In the first step, 4.5 mM of ARS in 0.1M sodium phosphate buffer, pH 7.0, was prepared and 5 mL of the freshly prepared ARS solution were added to each membrane disc and left to react for 20h at room temperature, in a Heidolph Titramax 1000 Plate Shaker under 200-300 rpm. The reaction is necessary to guarantee the binding of the ARS dye to the phenylboronate immobilized ligands. In the second step, functionalized and ARS-stained membranes were washed with 10 mL of the buffer solution in order to remove any excess unbound ARS from the membrane surface. The absorbance of the membrane washing solutions was monitored spectrophotometrically at 500 nm for the quantification of ARS. This washing procedure was repeated until a constant absorbance of the washings has been reached. In the third step, bound ARS was eluted from the membranes of lot 1 using 5 mL of a buffer solution containing 0.5 M D-sorbitol, which actively competes with ARS for the phenyl boronate ligands. The eluent was replaced batchwise, with fresh eluent portions, each for 5 min during the first hour and thereafter for longer periods of time. For membranes of lot 2, bound ARS was eluted from these membranes using 2 mL of a buffer solution containing 0.5 M D-sorbitol. The eluent was replaced batchwise, with fresh eluent portions, each for 10 min to complete a total volume of 20 mL of D-sorbitol containing (100 mM) PBS solution was used for the elution process that took a total of 120 min.

The elution of ARS and absorbance measurements of the colored eluate (500 nm) were carried out until the membranes became colorless. The absorbances were used to calculate the amounts (mmol) of the eluted ARS dye using the a calibration curve experimentally obtained for ARS in the solution $[\text{ARS}] = 3919 \text{ M}^{-1} \text{ cm}^{-1}$.

2.5. Membrane chromatography

Before starting chromatographic races, the fluid channels in the chromatographic equipment were washed with MilliQ water until conductivity measures reached low and stable values. Then, the system was equilibrated with appropriate buffer. The two derivatized membrane discs were placed in a Ø 25mm stainless steel inline filter holder from Pall. The filter holder was connected to an AktaTM Purifier FPLC system (GE Healthcare, Sweden) that continuously monitored the conductivity and UV absorbance at 260 nm for nucleic acids and 280 for protein. The chromatographies were operated at a constant flow rate of 1 mL/min and at room temperature; still in these experiments two buffers were used: the binding and washing buffer and the elution buffer. In the IgG chromatographies, the buffers were 20mM Hepes, for binding, and 1.5 M Tris-HCl for elution. Then, on first trials with IgG solutions, 100µL samples of pure IgG (1.0 g/L) were injected while running the binding buffer. During the elution, the eluted fractions were not collected. In the second trials, with IgG (also later for pDNA-containing cell lysate samples) 500 µL feed volumes were injected while running the binding buffer. During the run, 500µL fractions were collected from the flow through and eluate, which were frozen and maintained at -20°C until analysis. At the end of each run in the Akta™ system, the chromatograms were automatically generated and recorded.

2.6. Agarose gel electrophoresis

The protocol of agarose gel electrophoresis used in this work was the system composed by a GE Healthcare/Amersham Pharmacia Electrophoresis EPS 3501 XL Power Supply and one submarine electrophoresis unit a Hoefer HE 99 for the 20 cm long gels. The sample fractions collected from chromatographic runs during pDNA purification from clarified lysates, both from flowthrough and elution profiles, were analysed by electrophoresis in a 1% agarose horizontal gel (SeaKem® LE Agarose) in 1x TAE solution. The agarose gel electrophoresis used in this work was the system composed by a GE Healthcare/Amersham Pharmacia Electrophoresis EPS 3501 XL Power Supply and one submarine electrophoresis unit a Hoefer HE 99 for the 20 cm long gels. The sample fractions collected from chromatographic runs during pDNA purification from clarified lysates, both from flowthrough and elution profiles, were analysed by electrophoresis in a 1% agarose horizontal gel (SeaKem® LE Agarose) in 1x TAE solution - 40mM Tris base, 20mM acetic acid and 1mM EDTA, pH 8.0. The gel was prepared by mixing 1.80g agarose LE with 180mL of TAE solution. This long gel was run at 112V for approximately 1h30min. Side by side with other samples, the HyperLader™ I from Bioline was also run as DNA molecular weight marker. After ending the electrophoretic separation, the agarose gel was stained with ethidium bromide (EtBr) (0.5 mg/mL) or GelRed™ and imaged and analyzed using a Stratagene EagleEye II Video Imaging System or a VWR GenoSmart gel documentation system, respectively. Digital images of the gels were evaluated qualitatively.
2.7. Polyacrylamide gel electrophoresis

For this analysis 2 gels were prepared: resolving gel and stacking gel. According to the instructions manual, each unit cell in which a gel would be prepared was assembled using gloves. The first, the resolving gel solution was carefully prepared by mixing of reagents: Acrylamide 40% (5.8 mL); Bis-acrylamide 2% (3.2 mL); 4x Resolving Buffer (5.0 mL); mill-Q water (5.8 mL); TEMED (0.010 mL) and APS (0.100). The TEMED reagent and ammonium persulfate were added just when the casting solution is ready to pour, then, a pipette was filled with 20 mL of the resolving gel was slowly poured into the caster until the solution reaches the necessary level (indicated by green bar). After, the stacking gel was prepared by mixing of reagents: Acrylamide 40% (0.48 mL); Bis-acrylamide 2% (0.26 mL); 4x Stacking Buffer (1.25 mL); mill-Q water (2.96 mL); TEMED (0.005 mL) and APS (0.025). Then, a pipette was filled with 5 mL of stacking gel was slowly poured over into the resolving gel in the caster until the solution reaches the necessary level. Gels were allowed to polymerize for approximately one hour. Remaining gel overlay solution was removed by blotting the top of each gel with a piece of paper. Sample wells and lanes were defined in each gel by carefully pressing an appropriate comb into it. After displacing the desired amounts of selected (IgG) protein-containing samples, when the samples were prepared by mixing IgG samples collected being 20 µL of each sample collected with 5 µL of TTP and 25 µL of buffer radio. Then, each solution was placed in an appendorf for agitation and further heating at 165°C. Afterwards, the desired amounts (20 µL) of selected IgG-containing samples, taken from each appendorf microtube, were displaced in the gels side by side with the Bio-Rad Protein Standards and the electrophoresis experiment was performed at 90 V, for approximately 2 hours. When the front of the gel reached the bottom of the plates, the power is turned off and the gels were carefully removed from the plates, for later staining with a blue dye reagent.

3. Results and discussion

3.1. The derivatization procedure

The first attempts to immobilize phenylboronate into membranes, when not using NHS was not efficient. The number of derivatives of boronics acids linked in each membrane specimen was small resulting in low yield of the derivatization reaction. In the second attempt, the derivatized membranes in the presence the NHS showed higher chromatographic peaks consequently higher reaction yields in the binding of phenylboronate.

3.2. Amine density

The amine quantification of membranes performed with TNBS method, shows that the reaction media used to produce the aminated membrane has a high yield (70-83%) which hypothesizes a high membrane capacity towards IgG and pDNA impurities since the following PB functionalization will depend on efficiency of this previous membrane preparation process. Besides that, the subsequent derivatization reactions through the amine moieties are also crucial to reduce the quantity of final residual amines since their presence is undesirable. The estimated density of surface amines value obtained of 0.07 µequiv cm⁻² (Table 1) showed that the amination reaction was effective and good (amination yield reached 70%), when compared with yields of of circa 22% for other approaches on amination process that were used before (starting from epoxy-activated Sartobind Epoxy 75 membranes) [10]. Thus, the value found in this work represents a good result, but can be improved with optimization study of this amination step. However, former work at Institute for Biotechnology and Bioengineering, Instituto Superior Técnico, Lisboa, used epoxy-activated membranes that had pre-activation density of 1.5 µequiv epoxy cm⁻². Thus, the value founded in this work represents a good result, but can be improved with optimization study of this amination step.

Table 1. Alkali-released amine concentration and amine surface density obtained by the TNBS method after the first derivatization step.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amine Concentration (mol/L)</th>
<th>Amine surface density (µequiv m⁻²)</th>
<th>Aldeyde surface density (µequiv m⁻²)</th>
<th>Amine yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane control</td>
<td>----------------------------</td>
<td>---------------------</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>Derivatized membrane 1.1</td>
<td>2.3x10⁻⁵</td>
<td>0.07</td>
<td>0.03</td>
<td>70</td>
</tr>
<tr>
<td>Derivatized membrane 1.2</td>
<td>2.7x10⁻⁵</td>
<td>0.08</td>
<td>0.02</td>
<td>83</td>
</tr>
</tbody>
</table>

3.3. Accumulated amounts of alizarin

From the data obtained through the elutions was also possible to calculate the accumulated amounts of alizarin (indirectly, the amount of phenylboronate) on the surfaces of derivatized membranes. For the first lot of derivatized membranes, the membranes 1.3 via 1 and 1.4 via 2 showed a higher amount of accumulated alizarin mass and, indirectly, higher content of phenylboronate ligands, whereas the
membranes 1.3 via 2 and 1.3 via 1 showed the same amounts of the accumulated mass and therefore the same graph (Figure 3).

![Figure 3](image1.png)

**Fig. 3.** Representation of eluted alizarin mass on (A) derivatized membrane with 3-carboxyphenyl-boron acid way 1 and (B) on derivatized membrane with 4-carboxyphenyl-boron acid way 2.

For the second lot of derivatized membranes with NHS-mediation, the membranes that showed a higher amount of accumulated Alizarin mass, and indirectly of phenylboronate, were: 1.3 via 1 e 1.4 via 1 (Figure 4).

![Figure 4](image2.png)

**Fig. 4.** Representation of eluted alizarin mass on (A) derivatized membrane with 3-carboxyphenyl-boron acid way 1 and (B) on derivatized membrane with 4-carboxyphenyl-boron acid way 2.

### 3.4. Chromatographic experiments

According to the chromatograms of membranes from lot 1, it was observed that the PB-derivatized membranes that showed the highest values of chromatographic elution peak were the membranes: 1,3 via 1 and 1,4 via 2, which also showed higher adsorption of alizarin (the same higher accumulated mass) in adsorption experiments and therefore the same graph (Figure 3). In that way, we can infer that these membranes showed good adsorption of alizarin, but in this first stage of chromatographic experiments (flowthrough) presents a high amount of non-adsorbed IgG with theses membranes. Thus, the chromatographic performance of the membranes from lot 1 has not been as good as expected.

According to the chromatograms from lot 2, it was observed that the PB-derivatized membranes that showed the highest values of chromatographic elution peaks were the membranes: 1.3 via 1 and 1.3 via 2, which also showed higher adsorption of alizarin (the same higher accumulated mass) in adsorption experiments. These membranes showed good adsorption of alizarin and also were the ones that adsorbed more mAb, as in this first stage of chromatographic experiments, the flowthrough did not present non-adsorbed IgG peak. This means that these membranes showed possible capacity of adsorption / elution of pure IgG. Then, it can be affirmed that these derivatized at presence of NHS should contain largest amount of phenylboronate ligand on their surfaces which resulted in the best performances of these membranes analyzed in this batch, proving that the derivatization mediated by reagent NHS in a way increased the yield of the reaction.

According to chromatograms from lot 3 it was possible infer that the membrane 1.4 via 1, shows higher density of phenylboronate ligands on its surface, when compared to membrane 1.3 via 1, since the respective flowthrough had a smaller peak with a corresponding larger elution 1 peak on membrane 1.3 way 1 and a smaller peak with a corresponding larger delay peak on membrane 1.4 way 1. This also indicates that a higher derivatization yield was obtained in the chemical reaction process for membrane adsorber functionalization.

Moreover, chromatographic runs were performed with membranes 1.3 via 1 and 1.4 via 1, derivatized in the presence of NHS to adsorb/elute IgG antibody and 500μL of pure IgG antibody (1.0 g/L in 10 mL buffer) were injected in these PB-derivatized membrane adsorbers. This time, the samples were collected from flowthrough and eluted fractions for analytic gel electrophoreeses. The results from these experiments can be seen in the following Table 2.

### Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak name</th>
<th>Retention (mL)</th>
<th>Area (mAU.ml)</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td>Flowthrough</td>
<td>10.51</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>1,4 way 1</td>
<td>Elution 1</td>
<td>19.48</td>
<td>49</td>
<td>19.7</td>
</tr>
<tr>
<td></td>
<td>Delay</td>
<td>37.53</td>
<td>190</td>
<td>73.3</td>
</tr>
<tr>
<td>Membrane</td>
<td>Flowthrough</td>
<td>2.38</td>
<td>82</td>
<td>35.2</td>
</tr>
<tr>
<td>1,3 way 1</td>
<td>Elution 1</td>
<td>18.32</td>
<td>127</td>
<td>54.5</td>
</tr>
<tr>
<td></td>
<td>Delay</td>
<td>36.88</td>
<td>24</td>
<td>10.3</td>
</tr>
</tbody>
</table>

According to these results, the derivatized membranes with PB showed the best chromatographic performance, with the membrane 1,3 via 1 showing the highest retention and area values. However, further studies are needed to optimize the conditions and improve the chromatographic performance of these membranes.
For these chromatographic races of 500 µL of clarified lysate, the solutions used were MilliQ water, for adsorption, and 1.5 M Tris-HCl buffer, for isocratic elution, and the runs lasted 40 minutes at the flow rate of 1mL/min. The chromatographic areas obtained in these runs can be seen in the following Table 3.

Table 3. Areas subjacent to the peaks found in chromatographic runs with membranes 1.3 via 1* and 1.4 via 1* from the lot #3, for the adsorption/elution of impurities from 500µL of pDNA containing clarified lysates.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak name</th>
<th>Retention (mL)</th>
<th>Area (mAU.ml)</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td>Flowthrough</td>
<td>1.46</td>
<td>1555.8</td>
<td>87</td>
</tr>
<tr>
<td>1,3 way 1</td>
<td>Elution 1</td>
<td>17.94</td>
<td>222.3</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>Elution 2</td>
<td>38.57</td>
<td>10.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Membrane</td>
<td>Flowthrough</td>
<td>1.29</td>
<td>1214.6</td>
<td>77.8</td>
</tr>
<tr>
<td>1,4 way 1</td>
<td>Elution 1</td>
<td>18.04</td>
<td>333.9</td>
<td>21.4</td>
</tr>
<tr>
<td></td>
<td>Elution 2</td>
<td>38.26</td>
<td>12.3</td>
<td>0.8</td>
</tr>
</tbody>
</table>

It can be concluded from the results shown in Table 3 that the derivatized membranes were not able to adsorb the impurities and separate the pDNA in the 500 µL of clarified lysate, because a very large flowthrough peak is obtained and only very limited elution peaks were found. The reason for the unsuccessful experiments is probably due to not having enough phenylboronate ligands on the surface of membrane adsorbers (very limited capacity) or to an excess of injected lysate to carry out the chromatographic runs. The limit of capacity of the membrane was highly surpassed.

3.5. Analysis of gels

These analyses were performed to attempt qualitative characterization of samples taken from collected fractions from the chromatographic runs with clarified lysates and with pure IgG antibody solution. The feeds, flowthrough and elution fractions were analysed.

From the assays with two PB-derivatized membranes 1.3 via 1 and 1.4 via 1, the electrophoresis over the nucleic acid containing samples (Figures 5; gels revealed with EtBr), it was observed that the membranes were not able to adsorb all the RNA present in the clarified lysate. The expected result was that the pDNA bands would be clearly observable in the flowthrough lanes (below the wells), because pDNA is not supposed to bind to the PB-ligands. However, clearly visible sc- or oc-pDNA bands were only found below of the second well (lane F), that corresponds to the injected feed sample which also demonstrated to contain a lot of residual RNA (large band on the bottom of the lane in the gel).

Analyses were performed for the samples collected fractions of IgG from chromatographic runs with PB-derivatized membranes 1.3 via 1 and 1.4 via 1. The gels used in the experiment had 10 lanes each. Figure 6-part A characterizes fractions from the run with membrane 1.3 way 1, while Figure 6-part B characterizes fractions from run with membrane 1.4 way 1. After the procedures performed for revelation of the gels bands, the antibody chains were visible in the flowthrough and elution lanes. Looking at the Figure 6, in the gel lanes corresponding to "flowthrough" and elution, it is possible to visualize faint bands (from treated samples of collected chromatographic fractions). The bands at 80 kDa and 28 kDa were expected since they represent pure IgG antibody used in this analysis. The samples used in this experiment were too diluted and therefore it is difficult to analyze the results on the gel. It can be inferred from the results that possibly the membrane 1.4 way 1 will be better able, given that less residual IgG was seen on the gel, but such a may be only an artefact due to possible failure in its preparation because there was no repetition.

The results indicate that the IgG pure adsorption capacity of these PB-membranes was insufficient for the feed injected volume. None of the two PB-derivatized membrane specimens demonstrated to have at their surface the needed amount of phenylboronate ligand units, to promote the separation of this mAb.
Figure 5. Picture of agarose gels after electrophoretic separation of chromatographic fractions from injected clarified lysates (EtBr revealed) on (A) matrix 1,3 way 1. L – Molecular weight ladder; F – Feed; 1 – 5 Flowthrough and 6 – 12 Elution and (B) matrix 1,4 way 1. L – Molecular weight ladder; F – Feed; 1 – 5 Flowthrough and 6 – 10 Elution.

Figure 26. Picture of polyacrylamide gels after electrophoretic separation of chromatographic fractions from injected IgG solutions (Coomassie blue dye revealed) on (A) matrix 1,3 way 1. M – Biorad marker; 1 – 4 Flowthrough and 5 – 7 Elution and (B) M – Biorad marker; 1 – 3 Flowthrough on matrix 1,4 way 1; 4 – 5 Elution on matrix 1,3 way 1.

4. Conclusion

The Preparation, characterization and performance of chromatographic membrane with phenylboronate ligands for protein separation and purification of pDNA purpose was addressed in the dissertation. This approach seems to be interesting because it allows a better separation efficiency and increases the solute selectivity with minimal mass transfer by diffusion to the surface, thus increasing the yield of the process. Thus, the study of this thesis was work out to develop a membrane adsorbers pre-activated aldehyde groups through derivatization process based on two stages have been the activation of matrices with an aliphatic diamine and then the functionalization of these with boronic acid derivative using a carbodiimide coupling. This enables the ligands present in the membranes to promote affinity interactions with contaminants in pDNA-containing clarified lysates of E. coli and purify the pDNA during chromatographic techniques as well as with monoclonal antibodies (IgG) in cell culture supernatants to separate and purified thereof.

In a brief way, this thesis showed the importance of membrane separation process on purification of biomolecules and the importance of using membrane chromatography in biotechnology processing. Furthermore, it disclosed several chromatographic manners of purifying biomolecules by using two types of ligands, as ligand to target biomolecule interactions depend on both molecules properties, and evidenced that membrane adsorbers can be better for this purpose.
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6. References


