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Phenylboronic acid as ligand for multimodal chromatography

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Thesis approved in public session to obtain the PhD Degree in Bioengineering.
Pass with merit

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Uma vez disseram-me: “quando você terminar esse trabalho, nosso propósito na vida estará completo!” É certo que, para os pais, o propósito nunca está completo quando se trata dos filhos, porém digo a vocês: já estava mesmo quando comecei, pois o trabalho foi feito espelhado em seus ensinamentos: responsabilidade, respeito e honestidade! E assim sempre será! Obrigado pelo amor incondicional tendo a mim como parte do próposito de suas vidas!

À minha mãe e à memória de meu pai!

Resumo

Desde que a habilidade dos ácidos borónicos de formar ligações covalentes reversas com grupos *cis*-diol foi divulgada, vários estudos têm sido realizados com o intuito de explorar essa interacção. Entretanto, a interacção *cis*-diol é uma entre diferentes possíveis interacções do ácido fenilborónico. Esse comportamento multimodal é uma das principais razões pela qual os mecanismos de interacção dessa cromatografia ainda não estão completamente elucidados. Neste contexto estudos de caracterização das interacções entre o ácido fenilborónico e proteínas seleccionadas foram realizados em diferentes condições de adsorção com o objectivo de aperfeiçoar a selectividade dessa cromatografia de afinidade. Estudos de adsorção foram realizados numa faixa de pH entre 4 e 9 e diferenças na retenção das proteínas foram relacionadas com seu pI e presença de glicanos em sua estrutura. Estratégias de eluição foram então aplicados e boa selectividade foi obtida pela separação de glicoproteínas com gradiente de Tris e pela separação de proteínas ácidas/neutras com gradiente de citrato. Imobilizações de ácido fenilborónico em criogéis com grupos funcionais foram realizados em diferentes condições para posterior integração das etapas de clarificação e captura do processo de purificação de anticorpos. Todas as matrizes com fenilboronato mostraram ser capazes de adsorver IgG policlonal, porém selectividade e capacidade precisam ser aprimoradas para uma maior eficiência.

Palavras-chave: cromatografia fenilboronato, *cis*-diol, interação de afinidade, cromatografia multimodal, glicosilação, suporte monolítico, criogel, cromatografia líquida, anticorpos monoclonais, separação de proteínas

Abstract

Since the ability of boronic acids to form reversible covalent bonds with *cis*-diol groups was first reported, several studies have been performed to explore this interaction. Due to phenylboronic acid (PBA) multimodal behavior, *cis*-diol affinity is just one of the possible interactions between PBA and target molecules. This multimodal behavior of PBA is the main reason of miscomprehension of mechanisms of interactions of phenylboronate chromatography. Thus characterization of these additional chemical interactions between PBA and a selected protein library under different conditions was performed aiming the improvement of selectivity of phenylboronate chromatography. Adsorption experiments were carried out over a pH range from 4 to 9 and differences in the retention of these proteins were generally related with, charge (pI value) and presence of glycans. Elution strategies were also applied and a good selectivity was achieved when glycoproteins were separated through tris gradient and acidic/neutral proteins through citrate gradient. Immobilization of PBA, by different conditions, onto cryogels with functional epoxy group was also evaluated for further integration of the clarification and capture steps of the downstream process of antibodies. All phenylboronate matrices tested were able to capture polyclonal IgG, however selectivity and capacity of the process has still to be improved.

***Keywords**:* phenylboronate chromatography, *cis*-diol, affinity interaction, multimodal chromatography, glycosylation, monolith support, cryogel, liquid chromatography, monoclonal antibodies, proteins separation

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“I am not afraid of storms for I am learning how to sail my ship.”

Louisa May Alcott

Abbreviations List

3CPBA – 3-Carboxyphenylboronic acid

4CPBA – 4-Carboxyphenylboronic acid

ANS – 8-Anilino-1-naphthalenesulfonic acid

APBA – 3-Aminophenylboronic acid

ARS – Alizarin Red S

BA – Boronic acid

BAN – Buffer for acidic and neutral proteins binding at pH gradient

BC – Boronate chromatography

BDGE – 1,4-Butanediol diglycidyl ether

BGLy – Buffer for glycoproteins binding at pH gradient

CA – Carbonic anhydrase

CD – N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride

CHES – 2-(Cyclohexylamino)ethanesulfonic acid

CHO – Chinese hamster ovary

CV – Column volume

DBC – Dynamic binding capacity

DNA – Deoxyribonucleic acid

ED – Ethylenediamine

EG – Ethylene glycol

EPPS – 4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid

FPBA – Formylphenylboronic acid

GD – Glutaraldehyde

HEPES – 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid

HMD – Hexamethylenediamine

HPLC – High performance liquid chromatography

HSA – Human serum albumin

IgG – Immunoglobulin G

mAb – Monoclonal antibodies

MEP – 4-Mercaptoethyl pyridine

MES – 2-(4-Morpholino)ethanesulfonic acid

MMC – Multimodal chromatography

MOPSO – 3-Morpholino-2-hydroxypropanesulfonic acid

MPBA – Mercaptophenylboronic acid

MW – Molecular weight

PBA – Phenylboronic acid

PBC – Phenylboronate chromatography

PBS – Phosphate buffer saline

pI – Isoelectric point

RNA – Ribonucleic acid

RNase A – Ribonuclease A

RNase B – Ribonuclease B

RT – Room temperature

SEC – Size exclusion chromatography

TAPS – [(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid

Tris – Tris(hydroxymethyl)aminomethane

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CHAPTER I

GENERAL INTRODUCTION

General introduction

1. Background

The first boronic acid (BA) was isolated over 100 years ago, but only in the end of 50's the ability of BA to esterify with *cis*-diol compounds by reversible covalent bounds was identified [1]. Since then, several applications came up in different fields as pharmaceutical, biomedical and chemical [1,2]. The wide range of applications relies not only on the specific affinity interaction but also in the chemical stability and easy of handling making this compound very attractive [1]. The recent popularity of this acid is mainly due to two reasons: the first commercialized BA containing drug Velcade® approved as anti-cancer agent by FDA elevating the status of this chemical compound in chemistry and medicine and its application on receptors for saccharides since the first review demonstrating the potential of this application was published in 1996 which has attracted the attention of several research groups [1,3]. The application of BA in liquid chromatography started at 70's for saccharides and nucleosides separation [4,5] and at 80's studies for quantifying glycated hemoglobin from blood cells [6] was the first established process applied still used nowadays as a method for detecting diabetes in patients [7]. This chromatography has also been applied in several other diols separations including glycoproteins [8], catechol [9] and RNA [10] making boronate chromatography (BC) as an important separation tool specially for biopharmaceutical products [11].

Undoubtedly, all the BC purification process performed today bases on the esterification of *cis*-diol interaction, however the mechanisms of this interaction is still not well understood [12]. More recently, the classification of this ligand has been changed due to its ability to participate in multi interactions besides the *cis*-diol [13]. Phenylboronic acid

(PBA), the most used ligand in BC, has a phenyl moiety in its structure what brings a hydrophobic character to this chromatography as well as π interactions by the aromatic ring [13]. Furthermore, this acid can be found in two conformations due its ability to coordinate Lewis base molecules in conditions above its pKa, usually alkaline [1]. Under this condition, PBA coordinates free hydroxyls from medium becoming with a tetrahedral conformation and negatively charged [2]. Besides, hydroxyls from boron can also perform weaker hydrogen bond [13]. All these possibilities make this multimodal ligand not only a powerful separation tool but also a complex chemical compound and deeper understanding of how these interactions behave is crucial for developing efficient separation process, especially when the targets are complex biomolecules like proteins.

Among several applications, BC appears as a potential candidate for antibodies separation since these proteins are glycosylated. Antibodies are widely studied and applied in therapeutic treatments which are responsible for a market of billions of dollars [14]. The increased performance of cell culture processes has led to high titers of monoclonal antibodies over the last 10 years and the process bottleneck is now on the purification side. Efficient and selective processes are needed since the established capture process based on protein A chromatography has reached its capacity limit regarding the high production titers [15]. Moreover, this biological ligand has low stability and high cost reaching more than 50% of total cost of the process [8]. Our research group has made efforts to develop new alternatives for monoclonal antibodies (mAbs) separation and purification in order to replace protein A chromatography contributing in great extent to the scale-up of aqueous two phase extraction (ATPE) of mAbs via mixer settlers and packed-bed differential contactors which culminated in a join patent with Bayer Technology Services [16,17]. Currently, a microfluidic device was developed for the ATPE mAbs as an effective tool to accelerate bioprocess design and optimization [18]. In parallel, studies in liquid chromatography based on PBA were used for monoclonal antibodies purification

[8,19]. This ligand has higher stability and lower cost compared to protein A resins besides the specific selectivity with mAbs through affinity *cis*-diol interactions [19]. Different phenylboronate chromatographic supports have already been applied successfully for mAbs capture [8,19,20], although improvements on capacity and selectivity of the process are still necessary.

2. Thesis Scope and Outline

This work has been developed in the framework of MIT Portugal program in bioengineering systems at Institute for Biotechnology and Bioengineering of Instituto Superior Técnico in collaboration with Cramer research group at Rensselaer Polytechnic Institute in USA.

The main goal of this work is to investigate the various chemical interactions between proteins and PBA chromatographic supports in order to clarify all nonspecific interactions and understand their mechanisms in order to improve the performance of phenylboronate chromatography (PBC) for further applications.

The specific objectives comprise:

1. Identification of the dominant interactions between proteins and PBC by performing adsorption studies at different pHs ranging from 4 to 9 using a protein library based on their pI values and presence of glycosylation in their structures;
2. Evaluation of the matrix effect on PBC interaction by comparison of agarose and controlled-pore glass beads;
3. Evaluation of different elution strategies and modifiers in order to enhance PBC selectivity towards proteins;

4. Identification of the best binding condition for polyclonal IgG adsorption onto different PBC affinity matrices based on methacrylate and agarose, various pHs ranging from 7 to 9 and different buffer types (phosphate, HEPES and borate);
5. Immobilization of PBA by different conditions onto cryogels with functional epoxy group for further integration of the clarification and capture steps of downstream process of antibodies.

This thesis is divided in 7 chapters. Chapter II includes the literature review where is pointed out the important factors of affinity interaction of BAs and *cis*-diol compounds as well as the current applications of these acids. I also referred PBA ability to modulate different interactions and possible external conditions which could affect the interactions between this affinity chromatography and complex biomolecules as proteins. Chapter III presents the adsorption studies of different proteins to PBC chromatographic supports by using a protein library including glycosylated and nonglycosylated proteins in a wide range of pI values. The studies were carried out in a pH range from 4 to 9 covering trigonal conformation at acidic and neutral conditions and tetrahedral conformation at alkaline conditions. The comparison of different matrices was also performed using controlled-pore glass and agarose beads. As our knowledge, this is the first time that this fundamental work is performed with a protein library what we believe it will clarify and help several research groups interested in PBC applications. As a continuation of this chapter, chapter IV describes the use of same protein library for defining different elution strategies. The influence of several modifiers on PBC selectivity towards proteins was also analyzed. The elution strategies used are not only responsible for improvements of selectivity but also for clarifying the knowledge about mechanisms of PBA interactions. Chapter V focused on the application of PBC for antibodies capture. In this work, polyclonal IgG, HSA and human

insulin were separately loaded into PBC columns in order to analyze their adsorption under different binding conditions by varying buffer type and pH. Adsorption studies using two different commercial matrices based on methacrylate and agarose were also compared. This chapter also includes PBA immobilization onto cryogels supports in order to obtain functionalized PBA cryogels for process integration since this support provides large channels (100 μm) allowing the cells pass through and retaining the IgG. Different reactions were tried for cryogels functionalization with PBA and further analysis of ligand density and IgG binding were performed. Chapter VI presents the final remarks of this thesis summing up and concluding the main achievements of this work and future challenges. An appendix with extra information about protein library selection, PBC capacity and supplier information of the commercial supports used in this thesis is referred at chapter VII.

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CHAPTER II

PHENYLBORONATE CHROMATOGRAPHY: FROM AFFINITY TO MULTIMODAL BEHAVIOR

To Submit

Phenylboronate chromatography: from affinity to multimodal behavior

Abstract

The traditional classification of phenylboronate chromatography as an affinity technique has been changing in recent years. The ability of the ligand to engage in several interactions confers to this chromatography a multimodal behavior with electrostatic, hydrophobic, aromatic, charge transfer and hydrogen bond interactions playing an important role in addition to the affinity *cis*-diol esterification. Herein, the affinity and other interactions are pointed out by describing their importance. Examples of applications are also discussed as well as the relevance of multimodal behavior on them. From this work, an increase of interest on this unique molecule, phenylboronic acid, is expected especially for its implementation in bioseparation area.

Keywords: boronic acids, phenylboronate chromatography, bioseparation, glycoprotein, multimodal chromatography

1. Introduction

Boronic acid (BA) are typically alkyl or aryl substituted boric acids that contains a carbon–boron bond and two hydroxyl groups. BA has been reported as an Lewis acid [1], due to in the free p orbital of the boron atom which enables it to coordinate Lewis bases by charge-transfer interactions [2,3]. Although boronic acids are not found in nature, they can be easily obtained by second oxidation of boranes [1]. They are chemically stable molecules since oxidation by substitution of the carbon or hydroxyls from the boron atom is thermodynamically unfavorable [4,5], On the other hand, their physico-chemical properties

are highly dependent on BAs substituent group which can be responsible for changes in their acidity values for instance [1].

Although the first synthetic isolation of BA has been reported in 1860, the specific interactions performed by these acids came up in 1959 from Lorand and Edward studies [2]. According to their work, the neutral form of BA (trigonal conformation) is in equilibrium with the anionic form (tetrahedral conformation) due to its ability to ionize water and perform proton transference when the pH of the medium is at BA's pKa or above it (Figure 1). These authors also showed that BAs can perform reversible covalent bonds with *cis*-diol groups.

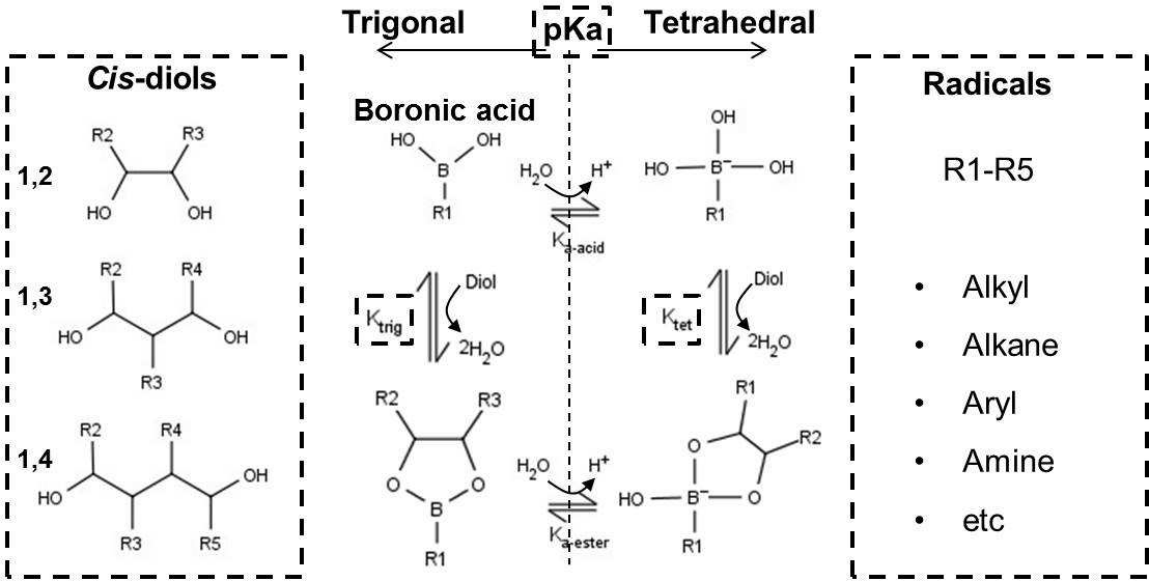

Fig. 1: Conventional map of BA esterification with *cis*-diol compounds. Molecules harboring *cis*-diol groups (1,2-, 1,3- and 1,4-*cis*-diols) and BA radicals in the dashed boxes, left and right, respectively. The two conformations are pH dependent with trigonal BA configuration prevailing at pH values below the pKa and tetrahedral configuration predominating at pH values above its pKa, usually basic. The equilibrium constants (K) for each reaction are also presented [6].

Lorand and Edward showed an increase in the acidity of BA when polyols were present in aqueous solution due to the formation of a cyclic ester [2]. These studies were the base of many other studies in this complex formation in the last four decades [3,6,7]. Several works performed on this equilibrium have shown higher association constant value when BA is at tetrahedral conformation. According to Hall (2005) [1], this might be associated to a transient state which involves charge-transfer and hydroniums displacement in the stability of boronate ester which results in a release of angle strain with tetrahedral hydrated form. However, the higher affinity above pKa value of BA is not an entirely true rule. Some studies have demonstrated that higher association constants at pHs lower than the pKa can be obtained and are dependent on the diol compound itself (Table 1) [6]. This hypothesis was also confirmed two years later by Yan et al. (2004) [8], who tried to correlate the pKa of both PBA and diol compound in order to predict the optimal pH for esterification. Accordingly, pKa values are important but not the only variant in this complexation. Another reason for this fact could be related to the radical of the *cis*-diol compound and its physico-chemical characteristics. For example, Alizarin red S (ARS), a red organic dye containing a 1,2 *cis*-diol group, has been reported to have a high affinity towards PBA. The equilibrium constant of ARS and phenylboronic acid (PBA) increases with the pH until an optimum is reached around neutral pH (Table 1). The decrease in affinity is probably related to electrostatic repulsion since both PBA (pKa 8.8) and ARS (pKa 6.94) are negatively charged in alkaline environments. Many efforts in order to disclose any relation of binding constants to pKa and pH of the medium has been recently performed. Martínez-Aguirre and his group [9] have recently proposed an equation for stability constants of boronate ester complexation based on acid-base properties using the Hammett- and Brönsted-type correlation. According to these authors, a comparison between calculated and experimental results could contribute to clarify the

external effects caused by steric conditions and additional bond formation. These effects have already been mentioned by Springsteen and Wang studies [6].

Furthermore, not only the external conditions can affect the affinity binding of boronate ester formation but also the substitute radical of both *cis*-diol and boronic acid. According to the literature, 1,2-, 1,3- and 1,4-*cis*-diols are able to complex with BA to form boronate esters [3]. The 1,2 *cis*-diols, which are able to perform five-members boronate esters, have been reported to have higher stability due to faster rate constants compared to six and seven-members boronate esters formed by 1,3- and 1,4-*cis*-diols complexation, respectively [1]. In addition, complex by tridentate interaction, when the reaction involves a chelating agent with three reacting groups, can likely occur. Tris(hydroxymethyl)aminomethane (tris) has been reported as the best shielding reagent for liquid chromatography among several others reagents able to perform tridentate interaction with PBA [10]. Although this interaction is not the strongest compared to 1-2 *cis*-diol compounds, it has high efficiency in order to avoid secondary interactions. This also might be the reason for the widely use of PBA in several applications for separation of diols compounds [11-14], protection of diols or diamines [15,16] and sensors for saccharides detection [3,17]. The structure effect on binding constant is currently being used as strategy in order to enhance the binding and stability of this affinity interaction by using substituents in arylboronic acids [9,18].

Table 1: Association constants (K_{eq}) of esterification of different diol compounds with PBA at different pHs. Values obtained from Springsteen and Wang (2002) [6].

pH	K_{eq} (M^{-1}) association constant of esterification PBA and diol					
	Fructose	Glucose	Galactose	Sorbitol	Catechol	ARS
6.5	29	0.84	2.1	47	150	1200
7.0	92	2.0	8.4	160	500	1500
7.4	160	4.6	17*	370	830	1300
8.0	310	7.2	38	840	2900	670
8.5	560	11	80	1000	3300	450

*pH 7.5

In 1994, a study from Shinkai and his group [19,20] has firstly showed the great potential of BAs as receptors in the development of sensing platforms for sugars and related saccharides using colorimetric arrays, which was in fact confirmed some years later by the increase of interest of several groups around the world [3,17,21]. Not only boronic acid based sensors, but other applications came out or were enhanced along with better understanding of BA reactions. Promoter of reactions such as i) catalyst reagent [22,23], ii) support for bioconjugation [24], iii) labeling of proteins and cell surface [25,26] and iv) enzyme inhibitor [27] are some examples of these applications. The fourth example was the first application of this class of acid, it has been applied for more than 70 years [28] and it is still used nowadays [27]. More recently, applications in the medical and pharmaceutical area have been also responsible for the growing interest in BA. Drug delivering by exploring the amphiphilic property of BAs in order to facilitate the transport of diol molecules into the cells is one of current researches as well as agent therapy for cancer [29]. In the early 2000, a drug for proteasome inhibitor has been approved by FDA and according to researches, the presence of PBA in substitution of an aldehyde from its structure was responsible for a substantial increase in potency of this drug [30]. This

successful example has inspired several scientists, and many studies on enzyme inhibitors BA-based came out. Currently, we can find drugs in combination with other therapies, for instance, using PBA in nanoparticles for drug delivery as therapy for liver cancer [31].

Due to the specificity of BAs affinity interaction with *cis*-diol groups, this acid became also a successful affinity ligand for capture of *cis*-diol content molecules including saccharides [32], nucleosides [12], ribonucleic acid (RNA) [33] and glycoproteins [11,14]. Although liquid boronate chromatography is widely studied nowadays, one of the first applications in this area was performed by gas chromatography [34]. Within the possible applications of boronate chromatography, glycosylated compounds separation is the most studied, for instance this separation method has already been largely applied for hemoglobin level measurement in red blood cells, an indicator of diabetes in patients [35,36]. Despite the establishment of this boronate chromatography separation process, there are still questions about the mechanisms of BAs complexation. Based on that, this literature review chapter addresses the fundamental studies of this affinity ligand for liquid chromatography with the main goal of clarifying the important questions about interactions and how to improve purification factors, mainly selectivity, when this chromatography is applied.

2. Boronate chromatography

Boronic acids have been applied in bioseparations for more than 50 years, however it was only in the 80's that their use was most widespread [1] and since then a wide variety of separation techniques using PBA have been described. Examples such as gas chromatography, the pioneer method as discussed before [34], extraction [13], incorporation in polyacrylamide gel SDS-PAGE [21] and capillary chromatography [37] can be found in the literature. Even with this large scenario of applications, liquid

chromatography is the most exploited separation method for analysis or process separation and has been classified as specific affinity ligands for *cis*-diol molecules, especially glycosylated proteins [1,38,39].

This affinity chromatography has been evolved regarding to the knowledge of BA interactions, but due some remains doubts about its mechanisms, several current studies using PBA support have discussed how the different conditions interfere on diol biomolecules retention in order to improve the performance of this chromatography [11,14,40]. Since this acid can change its conformation according to pH in the medium, the interactions also become pH dependent. Due to PBA ability to interact by different ways, it can be classified it as mixed-mode ligand [38,41]. The interactions of PBA can be predicted by its chemical structure and conformations (Figure 2), however the interactions do not act alone what makes unpredictable the way these interactions work together. Current studies in mixed-mode ligands have shown their high dependency on protein surface properties. Studies performed with hydroxyapatite liquid chromatography, a classic example of mixed-mode chromatography, have showed after analysis by quantitative structure property relationship (QSPR) models a synergistic binding with both interactions: metal chelation and cation exchange, which play a vital role for the binding of proteins [42]. Although multimodal chromatography can be a complex system, it can provide important advantages such as improvement of selectivity. Becker et al. (2009) [43], using proteins with small variance in hydrophobic amino acids substitution, characterized the ability of a multimodal hydrophobic interaction chromatography HIC-pH media. This multimodal chromatography possess three functional groups which combine hydrophobicity with pH-responsiveness and according to these authors, HIC-pH was able to selectively separate proteins with small variance in their structure proving a superior selectivity than conventional HIC media. In case of phenylboronate chromatography (PBC), alkaline conditions are required in order to obtain high retention of glycosylated proteins. At this

condition, negative charge of the tetrahedral BA conformation was found to affect the binding of glycoproteins. Azevedo et al. (2010) [11], obtained high retention of polyclonal IgG when studying their adsorption at pH 5.5 to 8.5, showing that even in the trigonal conformation, PBC was able to retain IgG thus highlighting the importance of mixed-mode interactions. These authors have found pH 8.5 as the best condition for IgG capture. Although this condition is still alkaline compared to other antibodies separation process, this condition was the most selective. In an opposite scenario, Zhang et al. (2009) [40] had lower retention at alkaline pH when studies with recombinant humanized IgG were performed due electrostatic repulsion between PBA and IgG which required NaCl at binding condition in order to mitigate the repulsion and obtain higher adsorption of this protein.

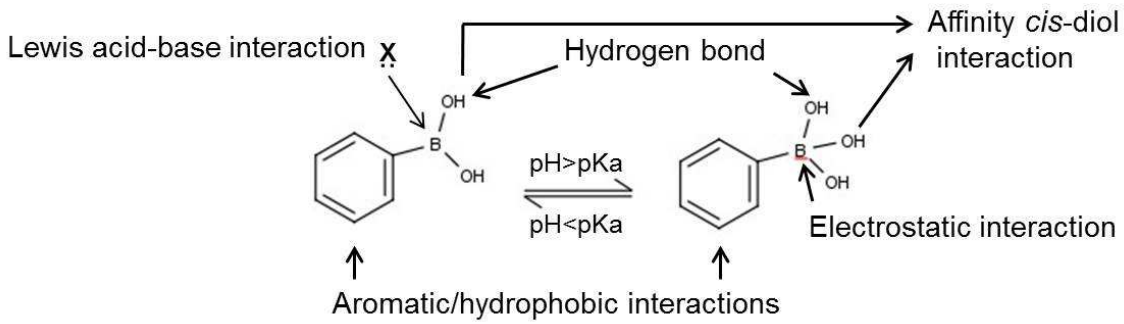

Fig. 2: Representation of PBA in two different conformations, trigonal at pH lower than its pKa, and tetrahedral in an opposite condition. From these two conformations, all possible interactions are described exposing the multimodal behavior of this acid: Aromatic/hydrophobic, electrostatic, hydrogen bond, Lewis acid-base and affinity *cis*-diol interactions. X is a Lewis base compound with an available pair of electrons to share with Lewis acid boron.

The multimodal characteristic of PBA is not only related to the ligand itself but sometimes to the chromatographic media composition and variations of the ligand, i.e. BA as ligand.

Different media composition can be found nowadays and depending on the chemical structure, an extra effect can be obtained. The choice is related to the purification process to be performed where the extra effect is likely favorable. Agarose, cellulose, methacrylate and glass beads are some examples of different matrix composition. For instance, agarose and cellulose can provide higher hydrogen bond interactions due to the high number of hydroxyls from their composition, on the other hand the hydroxyls from silica present in the glass beads become unprotonated and negatively charged above their pKa values [55].

Different variations of BA have been studied as well. These attempts were related to enhance the affinity interaction by increasing the association constants obtained with phenylboronic acid. Although an effort has been made in this direction, PBA is still the most used ligand in boronate chromatography [11,12,33,40,41].

2.1 Multimodal interactions of PBC

As previously described, the multimodal behavior of PBA can be easily predicted if the interactions are separately analyzed. They can be described by the two possible configurations which are pH dependent, however in both configurations the phenyl moiety can have some influence by aromatic interactions (hydrophobic, π-π and cation-π) or hydroxyls of boron by hydrogen bonding formation (Figure 2). At tetrahedral conformation the affinity by *cis*-diol is the primary interaction however the PBA becomes negatively charged and can act as cation-exchanger. On the other hand, the trigonal form is neutral and due free p orbital of boron, Lewis bases compounds can interact by charge-transfer [38].

2.1.1 Tetrahedral configuration behavior

Tetrahedral configuration is the target when PBC is applied [12,33,44]. As discussed in the first section the binding constant of this affinity interaction with cis-diol compounds is usually higher for the tetrahedral configuration than for the trigonal one, in aqueous medium. Alkaline condition is required in order to hydroxylate a trigonal conformation to tetrahedral since a pH equal or above PBA pKa is necessary. PBA, the most common BA applied in boronate chromatography, has a pKa 8.8 and the binding conditions for this ligand are usually applied above 8 [1]. Several studies have been performed regarding the esterification of PBA mostly with 1,2 *cis*-diol compounds [3]. Springsteen and Wang (2002) [6] reported several association constants of diol compounds with PBA at neutral pH, however these values increase about 4-fold when the pH was raised to 8.5 confirming the higher affinity as soon as the pH approximates to pKa value of PBA, except for ARS (Table 1). These values have been reported to be relatively weak compared to other affinity ligands, specially the biological ones such as lectin [45] or protein A [46]. On the other hand, PBA provides important advantages over protein A or other biological ligands as higher chemical stability and lower cost [44]. In addition, the affinity interaction of PBA can be very selective under certain conditions being compared with other affinity ligands [47].

Although the affinity *cis*-diol has been reported as primary interaction, the ability of the tetrahedral conformation to act as cation-exchanger can play an important role in the PBC, as already mentioned. For instance, the negatively charged PBC can strengthen the affinity interaction with positive patches of the biomolecule but also repel negatively charged biomolecules as well [40]. Nevertheless, this extra effect could be used as strategy for increasing selectivity of PBC. The phenyl moiety (further discussed at section 2.1.3) and the hydroxyl groups can also provide hydrophobic/aromatic and hydrogen bond

interactions, respectively. Hydrogen bond is a weak interaction and the probability of playing a dominant interaction can be low since two hydroxyls are esterifying and the possible hydrogen bond can only be performed by the third hydroxyl.

2.1.2 Trigonal configuration behavior

At neutral or acidic conditions, i.e. at pH values below BAs pKa, the trigonal configuration is obtained and there is a vacant p orbital capable to coordinate with other Lewis bases creating other interaction based on charge transfer where an electron pair from Lewis base can be coordinated by BA, a Lewis acid [1]. This interaction has not been much explored for separation purposes, however some authors have pointed charge transfer interaction as a reason for the binding of nonglycosylated proteins during purification [13]. Studies with human monoclonal antibodies (mAbs) from Chinese hamster ovary (CHO) cell culture supernatants using different supports with PBA immobilized provided high yields of mAbs, however insulin and human serum albumin, the main impurities in this culture, were also adsorbed by PBC specially when a neutral pH was applied in binding conditions [11,44]. According to these authors, available non protonated primary nitrogen from amino acids could be responsible for secondary charge transfer interactions since the trigonal conformation is dominant under this condition. In addition, carboxylic acids also present in acidic amino acid are negatively charged under this condition what classify them as hard Lewis bases [48]. These authors also described the hard Lewis bases as high electronegative compounds what would easily share their extra electrons.

The effect of some Lewis bases compounds, usually used as buffers, on association constants of BAs esterification with saccharides (fructose, mannitol, sorbitol and glucose) has been a studied in a wide pH range from 2 to 12 [7]. According to these authors a ternary complex (PBA/diol/Lewis bases) can be formed in a significant way and

be a dominant complex depending on the solution composition. This work also discusses the hardness of different Lewis bases compounds such as imidazole, phosphate and citrate. Citrate was found to be the hardest between these Lewis bases since this compound was able to better coordinate with PBA in a wider pH range. According to these authors, this behavior is probably related to the two possible ways of interaction, one by the usual charge transfer since citrate has three carboxylic acids in its structure, and a second by a chelate cyclic interaction with binary complex (PBA/Lewis bases) since citrate is also a hydroxyacid. However, the chelate interaction does not happen with ternary complex (PBA/diol/Lewis bases) [7]. In some situations, the Lewis base can even be responsible for cleaving the B-O interaction, depending on the strength of the Lewis base towards BA. Fluoride is an example of this compound and this strong interaction has been explored in order to use organoboron compounds as Lewis acid receptors for fluoride anions in acidic conditions [49]. The Lewis acid-base interaction is probably the dominant interaction when PBA is in the trigonal conformation, and thus the binding of biomolecules can likely be driven by charge transfer under certain conditions.

This conformation also allows for mixed-mode behavior through aromatic interactions by phenyl moiety (further discussed) and hydrogen bond by hydroxyls of PBA. Hydrogen bond is usually a weak interaction, and thus may only help stabilizing the main interaction rather than being the main precursor [50], especially for bigger biomolecules as proteins and RNA.

2.1.3 Phenyl moiety effect

PBA is the most used ligand for boronate chromatography [38]. The phenyl moiety of this ligand could also be responsible for strengthening the primary interaction providing hydrophobicity or aromatic interactions. In order to induce hydrophobicity, high concentration of salts are usually used in order to promote the “salting out” of biomolecules

[51]. However, PBC is usually performed in a low salt and buffer concentration in order to obtain low ionic strength and avoid hydrophobic interactions [52]. Nevertheless, aromatic cation-π and π-π interaction can still occur between the phenyl moiety of PBC and biomolecules. The presence of π-π interactions in PBC has been discussed for more than 20 years by several authors especially when it is applied in protein purification [40,44,52]. Brena et al. (1992) [52] showed in their study the Mg^{+2} effect on adsorption of immunoglobulin and suggested that it could be related with the π system since this cation was able to redistribute the π electrons what nowadays could be called cation-π interaction [53]. More recently, Zhang et al. (2009) [40] realized that non-specific interaction have occurred between the nonglycosylated heavy chain of recombinant human antibody and PBC and according to these authors π interaction could be responsible for the binding, although the interaction was not disrupted in the presence of phenylalanine. Nevertheless, these interactions are still only hypothesis and much more has to be elucidated.

Although the phenyl moiety does not suffer any structural changing between the two PBA conformations, some differences in chemical properties can be obtained. For instance, the additional polarity of tetrahedral form with 3 hydroxyl and negative charge around boronate could probably diminish hydrophobic interactions [38]. At the trigonal conformation the effect of the phenyl moiety could be more relevant although low ionic strength is still applied for PBC when this condition is used [13]. Even though, this conformation provides lower polarity increasing the probability of interactions with the phenyl moiety. This additional effect by phenyl moiety could bring nonspecific interaction but also higher selectivity to this chromatography, for instance, playing with salt concentration could be a strategy for enhance the selectivity of PBC for separating biomolecules by *cis*-diol affinity or Lewis acid-base interactions since these interactions are probably not salt dependent because of the covalent bond [1, 49].

2.2 Media, supports and ligands for boronate chromatography

The large popularity of PBC nowadays is responsible for the wide range of different media and supports options commercially available (Table 2). Additional interactions can be provided and somehow modify the multimodal behavior of this ligand depending on the media used for immobilizing PBA. ProSep®-PB, a controlled-pore glass (CPG®) beads with aminophenylboronic acid (APBA) functionalized has been applied in separation studies [11,54]. This media is a silica based material and when it is in pH above 6, hydroxyls can lose protons and become negatively charged acting as cation exchanger [55]. This feature can enhance the cation exchange behavior of PBA at the tetrahedral conformation, which is usually obtained only at alkaline conditions. Furthermore, CPG® is a hydrophilic media commonly used as a stationary phase in hydrophilic interaction liquid chromatography (HILIC), which increases the polarity and could diminish even more the phenyl effect of PBC. Besides the media, each support has also particular feature which characterize it for specific separations. For instance, several studies have been done in order to obtain monoliths PBA functionalized [47,56]. Srivastava et al. (2012) [56] has developed a monolith cryogel with vinylphenylboronic acid and according to these authors, the macroporosity of this support can be properly applied for larger biomolecules separations like cells, RNA and proteins. Monolithic supports have been widely applied on PBC in the last few years. Fine tuning monoliths [14], hybrid boronate monolith [57] and capillary chromatography [58] are examples of this application among several other attempts in production of monoliths by different monomers reactions and PBA derivatives [59].

Table 2: Examples of different media and supports used in PBC, commercially available or production described in the literature.

Material base	Supplier or reference	Support	Applications
Agarose	ProMetic	Beads	[52,60]
	Sigma-Aldrich	Beads	
Polyacrylamide	Bio-rad	Gel	[61]
	[56,59]	Monolith cryogel	[56]
Polymethacrylate	Tosoh	Gel (HPLC column)	[40]
	Sigma-Aldrich	Gel	[62,63]
Controlled-pore glass	Millipore	Beads	[11,54]
Magnetic particles (silica base)	Chemicell	Beads	[41]

New ligands for boronate chromatography have been explored as well. In the early 90's, studies with different variations of aliphatic boronate ligands using cellulose or agarose as matrices were performed [64,65]. Among these variations, some ligands had a hetero atom coordinated with boron (Figure 3) in order to create a tetrahedral conformation of ligand. This strategy favored the esterification with *cis*-diol compounds and it could also lower the pKa to 7.0 that would allow their use in this chromatography at neutral pH. However, none of these ligands were stable or efficient providing low ligand density or low association constants of *cis*-diol esterification. Some aliphatic ligands were not even able to interact with cathecol, an organic diol compound which has one of highest equilibrium constant with BAs (Table 1) [38]. Despite these different attempts, none of them provided better performance than PBA. Most of the current commercial available supports (Table 2) and studies with new supports for boronate chromatography use PBA as ligand. Current strategies are aiming not to the design of new ligands but of different functional groups for

immobilization in order to obtain higher immobilization yields and consequently higher ligand densities [59].

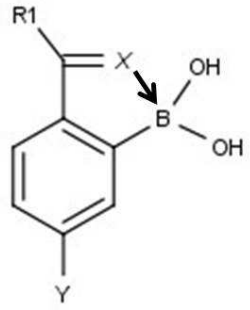

Fig. 3: Boronate ligand with internal coordination bond. X= O, N, S, etc. Y= -CH_2Br, -NH_2, -NCS, etc. R1= -$N(C_2H_5)_2$, -$N[CH(CH_3)_2]$, -OCH_3, etc [65].

2.3 Biomolecule surface properties effect on PBC

Besides the effect of geometrical structure of *cis*-diols in the esterification, the multimodal behavior can also be responsible for retention of biomolecules which includes many other possible interactions which are related to the exposed chemical surface of the biomolecules towards PBA.

Glycosylated proteins became one of the main targets of PBC. This class of proteins has been widely investigated in the last 20 years due their importance in transports of biomolecules during biological production in the cell machinery [66]. The glycoprotein has a carbohydrate chain (pentose and hexoses saccharides, uronic acid and sialic acid) linked covalently to N or O of specific residues of the proteins and they are not part of primary structure of the protein [66,67]. This covalent link can be performed enzymatically (glycosylation) or not (glycation), the latter is usually obtained by longer exposure of proteins in environments rich in glycans [40]. Although both are covalently

linked to the protein, studies have shown a preference binding of PBC to glycated recombinant humanized monoclonal antibodies (rhmAbs) [40]. According to these studies, vicinal *cis*-diol from glycosylation sites would need to overcome high energy barriers (up to 12 kcal/mol) to obtain the co-planar geometry necessary to bind to PAB since these diol compounds cannot overcome barriers energies > 7 kcal/mol. However, other works have already demonstrated glycosylated proteins binding using PBC [68]. An actual discussion is more related to ability of PBC separate glycoforms. Lu et al. (2013) [14] in an attempt to separate sialyted from nonsialyted glycosylated proteins played with pH dependency of PBA in order to obtain higher selectivity toward this sub-class of glycoproteins.

Concerning the multimodal behavior of PBC, other surface properties of biomolecules can likely affect their binding during this chromatography. Hydrophobic patches and charges on the surface of the biomolecules could be responsible for different behavior in the interactions occurring. Studies have demonstrated the surface properties dependence in multimodal interaction. Holstein et al. (2012) [69] has showed by NMR spectra different binding sites for the same protein ubiquitin when applied to a weak (CM sepharose FF), a strong (SP sepharose FF) and a multimodal (Capto MMC) cation exchange ligand. In PBC, different sites of the target biomolecule could be responsible for its binding depending on the surface properties of biomolecules. As discussed before, due to the negative charge of PBC at the tetrahedral conformation, the charge of the target molecule can influence its retention. Despite lacking of studies in this matter, the same rule could work for hydrophobic patches on biomolecule surface, the higher number of these patches could increase the probability of interactions with phenyl moiety of PBC. Studies on extraction of diols using PBA showed some effect of hydrophobicity of diol radical on separation [13]. According to these studies, the higher the alkyl chain, the higher the extractability obtained.

The presence of Lewis bases in the target biomolecules structure has also demonstrated being responsible for interfering on PBC interactions. Studies performed with different nucleosides in different conditions revealed that protic conditions (acetonitrile/H_2O) was not favorable for nucleosides retention, however when 100% acetonitrile was used, the retention obtained was effected by the nucleobase rather than by the presence of *cis*-diol moiety [12]. It is a fact that using an organic solvent, the deprotonated nitrogen of nuclease can easily compete by charge transfer due to the low presence of hydroxonium, what does not occur in an aqueous medium. In a different scenario, carboxylic acids from proteins could easily act as Lewis bases in trigonal conformation of PBA and be retained by charge transfer. For instance, acidic and neutral proteins (pI of protein < pKa of PBA) could likely perform this interaction since they theoretically have a higher number of acidic residues.

3. Conclusions and future trends

In the last 5 years, applications on PBC have focused on monoclonal antibodies [11,44], studies with glycated hemoglobin [70,71], plasmid DNA purification [54], and several glycosylated proteins purification used in proteomics [14,68,72] for pharmaceutical and biomedical purposes. The liquid chromatography technique is used in these cases aiming separation, characterization, analysis and quantification and all of them are exploring the esterification of PBA with *cis*-diol as a main interaction. Several examples were described throughout the development of this chapter, and accordingly, PBC is a powerful technique due to the unique interaction provided by PBA ligand able to bind selectively *cis*-diol compounds. In addition, this affinity chromatography has shown also a multimodal behavior which increases its selective ability when right strategies are applied.

The recent applications reported in the literature are based not only in the selective feature of PBC but also in the higher stability, lower toxicity and lower cost compared to other affinity chromatography. As mentioned before, the replacement of the established protein A chromatography for monoclonal antibodies purification is largely discussed nowadays. Several developments in upstream conditions have improved the production titers and the capture step of mAbs purification became the bottleneck of the process requiring more efficient and stable ligands. PBC is a potential candidate in mAbs separation, the first application dates from 1992 [52] but only few years ago several efforts have been made in order to improve this process [11,40,44]. From the same reasons, studies on replacement of lectin chromatography by PBC for proteomic analysis have been performed [14]. Other successful PBC application is described by Gomes et al. (2011) [54], who used a negative chromatography to obtain the target product, plasmid DNA from *E. coli* lysate, at flow through step. High yield of 96% was obtained from this as well as significant removal of impurities, including host proteins (61.3%), RNA (61.5%) and genomic DNA (44.7%). Furthermore, the significance of a such good purification factors persisted even after scaling-up of the process [33]. In a different perspective, PBC has been used for analysis and separation of hemoglobin for more than 20 years [35]. Recently, studies using this chromatography have demonstrated the important clinical issues as the best conditions for storage of blood samples with hemoglobin A1c from patients in order to obtain stability of these cells for later analysis [70].

Several other successful applications of PBC could be mentioned, however it is important to note that some mechanisms of this affinity interaction are still not completely understood. Most of the reasons are related to the multimodal behavior of the PBA ligand and how all the possible interactions can affect each other. Lacking on comprehension of phenyl moiety and hydrogen bond from hydroxyls contribution to this chromatography could be a key for some explanations of non-specific interactions obtained in some

studies. Moreover, exploitation of the Lewis acid-base interaction at trigonal conformation targeting other classes of proteins could increase the range of selectivity and applications of this chromatography (further discussed in the chapter III). Finally, PBC proved to be a powerful separation technique and a better understanding of its mechanisms of interactions is the key for the improvement of its efficiency.

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CHAPTER III

PROBING LIGAND-PROTEIN INTERACTION IN PHENYLBORONATE CHROMATOGRAPHY

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Carvalho, R.J., Woo, J., Azevedo, A.M., Cramer, S.M:, Aires-Barros, M.R. Phenylboronic acid as ligand for a multimodal chromatography: characterization using protein library – Affinity, Vienna/Austria, 2013.

Probing ligand-protein interaction in phenylboronate chromatography

Abstract

In this work, a protein library containing both glycosylated and nonglycosylated proteins with a wide range of molecular weights and pI values was employed to investigate the various chemical interactions between proteins and phenylboronic acid (PBA) chromatographic supports. Adsorption experiments were carried out over a pH range from 4 to 9 and differences in the adsorption of these proteins were generally related with two main properties, charge (pI value) and presence of glycans. For proteins with an acidic or neutral pI, it was necessary to add 200 mM of NaCl above pH 8, when tetrahedral conformation is already obtained, in order to adsorb glycosylated proteins onto the resin. The salt ions likely shielded the electrostatic repulsion between the negative charged tetrahedral PBA and the protein surface. In this pH range, PBA also acted as a cation exchanger, adsorbing basic proteins via electrostatic interactions, which could then be disrupted with the addition of 200 mM NaCl. Below pH 8, PBA is in an uncharged trigonal form, unable to make the electrostatic and *cis*-diol interactions found at high pH. Proteins exhibited higher adsorption around their pI, suggesting that hydrophobic or π–π interactions from the phenyl moiety of PBA contributed significantly to the retention of the target molecule. Adsorption studies performed in the presence of a Lewis base buffer (citrate) showed that at pH 4 and 6, the adsorption decreased for both neutral and acidic proteins, indicating that Lewis acid-base charge-transfer interactions between PBA and the carboxylic groups of proteins could also be a significant factor in protein adsorption at lower pH. This study has demonstrated that PBA interacts with proteins via several modes of interaction, including group specific affinity *cis*-diol, electrostatic, charge transfer and hydrophobic interactions.

Keywords: Phenylboronic acid chromatography, glycoproteins, multimodal chromatography, Lewis bases, *cis*-diol groups

1. Introduction

Many studies on boronic acids (BA) have focused on their ability to interact specifically with *cis*-diol containing biomolecules through the formation of a reversible pair of covalent ester bonds [1-3]. This rather unique feature has been widely explored during the last 30 years in the development of carbohydrate sensors [4,5], extraction of *cis*-diol containing molecules [6], labeling of proteins and cell surfaces [7,8] and in the affinity chromatographic separation of biomolecules such as glycoproteins, glycopeptides, nucleosides and nucleic acids [9-13].

BA ligands can be found in two different stable conformations depending on the pH of the medium. Typically at neutral or acidic environments, i.e., below the BA's pI, the boronic acid adopts a trigonal conformation while at alkaline environments the ligand becomes hydroxylated and the resulting boronate anion adopts a tetrahedral conformation (Figure 1)[3]. This hydroxylation is possible since trigonal BAs exhibit a Lewis acid behavior due to the deficient valence of the boron atom which has a free p orbital that enables it to coordinate with basic molecules [14], as hydroxyls, resulting in a highly stable tetrahedral conformation which is negatively charged at high pH. This simple change in BA conformation changes its affinity for *cis*-diol compounds, resulting from slower dissociation from tetrahedral complexes than from trigonal complexes [2,15].

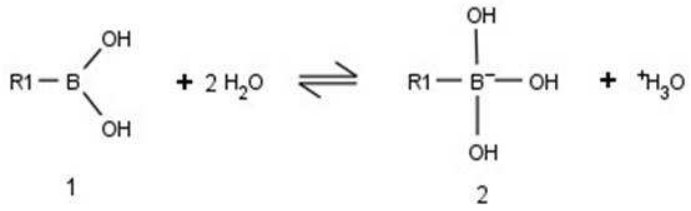


Fig. 1: Chemical equation of boronic acid ionization in aqueous environment according to Hall [14]. 1 – Trigonal conformation of BA. 2 – Tetrahedral conformation after ionization at high pH.

Phenylboronate (PB) chromatography has been broadly applied for glycoprotein separation and identification since it can specifically retain these proteins through binding the glycans present in their structures, especially at pH values higher than the pKa (8.8) of the PB ligand. Although the mechanisms of interactions are still not fully understood [16], current clinical applications such as glycated hemoglobin measurement are already being used [17,18] and other possible and potential applications have been suggested in the literature including the determination of glycan patterns [16,19] and the separation of complex biological mixtures [9,20,21]. This type of chromatography has important advantages such as low cost, high chemical stability and the specificity to replace established processes such as Protein A chromatography for monoclonal antibodies purification [9,21] and lectin affinity chromatography for isolation of glycoproteins for glycoproteomics [20]. Protein A and lectin affinity chromatography, although very effective due to their specific interactions, can have some drawbacks such as high cost and ligand instability [22,23].

In addition to the affinity interaction between BA and *cis*-diol molecules which is unique to PB chromatography, the Lewis acid character of the ligand also allows it to interact with hard Lewis bases whenever the pH is below the ligand pKa [24]. This secondary interaction could be exploited to increase the selectivity of *cis*-diol interactions

or as an orthogonal driving force for protein separations. Lewis acid-base interactions have already been explored in chromatography using zirconia as stationary phase. This support is also a Lewis acid which can interact with strong Lewis bases buffers and generate a negatively charged surface [25]. In PB chromatography, Lewis bases groups are present in proteins, in the form of negatively charged carboxylates (aspartate and glutamate) or unprotonated amino groups (asparagine and glutamine), and could easily interact with boron at lower pH [26]. Hydrophobic and aromatic π-π or cation-π interactions via the phenyl moiety are also likely playing a role, though these interactions are minimal at low ionic strength [27]. In addition, the hydroxyl groups can also establish hydrogen bonds with electronegative atoms on the protein surface [28]. Lastly, when the ligand is in the negatively-charged tetrahedral conformation, the hydroxyboronate anion can act as a weak cation-exchanger.

All the aforementioned secondary interactions are often disregarded by many authors that focus on separating molecules based on their *cis*-diol content [9,16]. Nevertheless, these secondary interactions should be taken into consideration as they can modulate *cis*-diol binding and even enhance both the selectivity and capacity of the chromatographic media as demonstrated in multimodal chromatography studies [29,30].

All of these proposed secondary interactions have a strong dependence on the solution pH. In this paper we characterize the adsorption behavior of a library of proteins containing glycosylated and nonglycosylated species with isoelectric points ranging from 2.5-9.5. Adsorption experiments were performed over a pH range of 4 to 9 in 96-well plates and in packed columns, in order to understand and categorize the possible interactions between PB and different classes of proteins (acidic, neutral, basic, glycosylated and nonglycosylated) and determine which interactions are dominant at different pH ranges.

2. Materials and Methods

2.1 Materials

The protein library was composed by the following proteins obtained from Sigma-Aldrich® (St. Louis, MO, USA): amyloglucosidase from *Aspergillus niger*, carbonic anhydrase from bovine erythrocyte, cellulase from *Trichoderma reesei*, invertase glycoprotein standard from *Saccharomyces cerevisiae*, pepsin from porcine stomach, ribonuclease A (RNase A) from bovine pancreas and ribonuclease B (RNase B) from bovine pancreas.

8-Anilino-1-naphthalenesulfonic acid (ANS), 2-(Cyclohexylamino)ethanesulfonic acid (CHES), sodium phosphate mono and dibasic anhydrous, sodium citrate anhydrous, sodium acetate anhydrous, sodium fluoride, tris(hydroxymethyl)aminomethane (Tris), sodium chloride and glycine were also purchased from Sigma-Aldrich. Ethanol was purchased from Panreac (Panreac, Barcelona, Spain) and glacial acetic acid from VWR (VWR, Ecuador). The chromatographic media used in this study were controlled porous glass (CPG®) and aminophenylboronic acid functionalized CPG® (ProSep®-PB, EMD Millipore, UK), Agarose P6XL (ProMetic Biosciences, UK) and pre-packed HiTrap™ Phenyl HP (GE Healthcare, Sweden). All reagents used had a purity ≥ 98%, were PA or of HPLC grade. All the water used in the experiments was deionized water.

Äkta Explorer and Purifier systems (GE Healthcare, Uppsala, Sweden) were used to perform all column experiments, both systems were operated online by the software Unicorn 5.11. Tricorn™ empty glass columns (i.d. 5 mm; max. bed height 109 mm) were used for packing ProSep®-PB and Agarose P6XL to a column volume of 2 mL (CV). Batch experiments were performed with MultiScreen™-HV 0.45 µm Durapore 96-well membrane plates (EMD Millipore, USA).

2.2 Adsorption experiments

2.2.1 Column experiments

Proteins were loaded in the column in amounts between 135 to 216 µg, lower loadings were performed in order to avoid saturation of the column. The pH range used in these studies was from 4 to 9 and the adsorption buffers used were i) 50 mM sodium acetate from pH 4 to 6, ii) 10 mM sodium phosphate from pH 7 and 8 and iii) 20 mM CHES for pH 9, with the addition of either 0 or 200 mM NaCl. Elution of adsorbed proteins was triggered by changing the eluent to 300 mM Tris-HCl, pH 8.5 under a 10 CV gradient elution. The column was cleaned with 30 mM acetic acid, pH 3. 2 mL columns of either CPG®, ProSep®-PB and P6XL were employed in the column experiments.

The percent adsorption of protein was determined based on area of the Tris-HCl elution peak divided by the total peak area in the chromatogram. Randomly selected conditions were duplicated and showed a standard deviation of 3% or less.

2.2.2 Batch experiments

In order to investigate the dominant interactions at the acidic/neutral pH range, batch experiments were performed in which the adsorption buffer was supplemented with different Lewis bases buffers or ethanol, with the intention to disrupt either charge transfer interactions using Lewis bases buffers and/or hydrophobic interactions and thus identify the dominant interactions for protein adsorption to PB. Pepsin, carbonic anhydrase and cellulase were used in these studies. The experimental conditions are listed in Table 1.

Table 1: Binding conditions of batch experiments and the expected effect of adsorption.

Buffers and modifiers	Binding conditions						Lewis base strength [26]	Mitigation	
	Concentrations (mM)					pH		Interaction	Effect expected
Acetate	10	50	100	200	200	4	Hard	Charge transfer	Low
Fluoride	-	50	-	-	-	4	Medium	Charge transfer	Low
Phosphate	10	50	100	200	200	6	Medium	Charge transfer	High
Citrate	10	50	100	200	200	4 and 6	Hard	Charge transfer	High
Ethanol*	20%	-	-	-	20%	4 and 6	-	Hydrophobicity	High
Glycine*	20 and 100	-	-	-	-	4 and 6	Hard	Charge transfer	Medium

*Ethanol and Glycine were prepared with Acetate at pH 4 and Phosphate at pH 6.

The adsorptions were performed in 96-well microplates with membranes, 200 µL of protein solution was added in 20 µL of ProSep®-PB and agitated for 5 h using shaker-incubator Stat Fax™ 2200 (Bio-Rad, USA) at maximum velocity and then the supernatant was vacuum into UV 96-well microplate and the values of samples at UV 280 nm was obtained with SPECTRAmax Plus 384 microplate reader (Molecular Devices, USA). The percentage of adsorption was calculated based on Eq. (1):

$$P = \frac{Ci - Cf}{Ci} \times 100 \quad (1)$$

where P is the percentage of protein adsorption, *Ci* the initial concentration of protein (mg/mL), *Cf* the concentration of supernatant obtained after adsorption (mg/mL). The experiments were performed in triplicate.

2.3 Ligand density measurement

The ligand density was determined using Alizarin Red S (ARS) adsorption to phenylboronic acid [31]. The assay was performed at pH 7 using ProSep®-PB and P6XL in 96-well membrane plates 0.45 µm using MultiScreen™ Vacuum Manifold 96-well

system for filtering dye solutions, as well as wash and elution steps. The concentration of dye used was 4.5 mM ARS in 100 mM of phosphate buffer pH 7 and three different resin:ARS ratios were tested: 1:4, 1:10 and 1:20. The resins were in contact with dye for 4 hours with the solution exchanged after 30, 60 and 150 minutes. After dye adsorption, the resins were washed several times with 100 mM of phosphate buffer, pH 7 until the solution was uncolored. Dye was eluted with 500 mM sorbitol in the same phosphate buffer until no remaining dye could be detected. The measurement was monitored by SPECTRAmax Plus 384 spectrophotometer microplate reader (Molecular Devices, USA) at λ = 500 nm.

2.4 Hydrophobic studies

2.4.1 Phenylsepharose chromatography

80 µg of Amyloglucosidase, Pepsin, Carbonic Anhydrase, Cellulase and RNase A and B were separately loaded in the HiTrap™ Phenyl HP 1 mL column. The runs were performed in isocratic mode over 10 column volumes (CV) using 50 mM acetate (pH 4 and 6) and 20 mM CHES (pH 9). After each run, columns were cleaned using 3 CV of deionized water followed by 3 CV of 20% ethanol. Additional experiments were also performed using 10 and 20 mM citrate buffer at pH 4 as binding conditions.

2.4.2 8-Anilino-1-naphthalenesulfonic acid (ANS) binding

Protein solutions (0.3 g/L) prepared in several buffers containing 7.5 µM ANS were incubated in a plate shaker for 30 min. The fluorescence of each protein solution was measured in a Varian Cary Eclipse Fluorescence spectrophotometer equipped with a microwell plate reader (Varian, Palo Alto, USA). Measurements were performed at room temperature and the emission spectra were recorded from 450 to 560 nm with excitation at 440 nm. The maximum values were obtained at 485 nm.

2.5 Protein surface properties analysis

The 3D structure files for each protein were obtained from Protein Data Bank (PDB) of Research Collaboratory Structural Bioinformatics (RCSB). All proteins were pretreated to remove solvents and glycans from the structure file and later protonated at the desired pH using the Molecular Operating Environment (MOE) 2010.10 software (Chemical Computing Group, Montreal, Canada) using the Amber99 force-field. After creating a structure file for each pH in the study, the protein surface properties (electrostatic potential (EP), spatial aggregation propensity (SAP) and charged amino acids maps) were visualized using PyMol 1.5 software (Schrodinger Inc., NY, USA). For SAP maps, the hydrogen atoms were deleted from the protein structure file before calculations were performed as described elsewhere [32] and the output was visualized using PyMol.

2.6 Analysis of Phenylsepharose chromatography fractions by size exclusion chromatography (SEC)

Analyses of fractions from Phenylsepharose chromatography were performed by SEC in HPLC system in order to analyze possible aggregation of them. Samples were run in isocratic mode in a TSK-Gel Super SW3000 column equipped with a TSK-GEL Super SW guard column, both from Tosoh Bioscience (Stuttgart, Germany) at 0.35 mL/min for 30 min in phosphate buffer saline (PBS) at pH 7.4. HPLC purity was quantified by the ratio of the IgG peak area and the total area of the chromatogram subtracted by the total area of the corresponding buffer chromatogram. The aggregation was analyzed by molecular weight of the samples measured from a calibration curve built with Bio-Rad protein gel filtration standards (Hercules, CA, USA).

3. Results and Discussion

3.1 Adsorption studies on packed columns

Seven proteins were selected as standards for the protein library among several proteins (Appendix A1). According to their pI, proteins were categorized as acidic, neutral or basic and each group contained both a glycosylated and non-glycosylated protein (Table 2 and Appendix A2).

Table 2: Isoelectric point (pI), Molecular Mass (MM), Protein Data Bank Identity (PBD ID) and classification within the protein library used in this work.

Protein	pI	PDB ID	Classification
Pepsin	2.6	3PEP	Acidic, NGly*
Amyloglucosidase	3.6	3GLY	Acidic, Gly**
Invertase	3.5	3KF5	Acidic, Gly
Carbonic Anhydrase	6.6	1V9E	Neutral, NGly
Cellulase	7.7	3QR3	Neutral, Gly
RNase A	9.6	1RBX	Basic, NGly
RNase B	9.7	1RBB	Basic, Gly

*NGly=Nonglycosylated **Gly=Glysosylated

These proteins were selected in order to understand the general behavior of proteins on phenylboronate (PB) chromatography in the pH range from 4 to 9. As described in the experimental section, two chromatographic resins with the same aminophenylboronic acid functionalization were used in this study; ProSep®-PB with a controlled pore glass (CPG) matrix (Appendix B1) and Agarose P6XL (Appendix B2) with an agarose matrix. Adsorption of nonglycosylated proteins on bare controlled pore glass, CPG®, was also studied as a control.

3.1.1 Basic proteins

The adsorption behavior of the basic proteins RNase A (nonglycosylated) and RNase B (glycosylated) were examined as shown in Figure 2. As can be observed in the figure, the adsorption of these proteins increased with higher pH, independent of the presence of glycans. Curiously, the onset of adsorption occurred at a lower pH value for the ProSep®-PB matrix (pH 5-6) than for the Agarose P6XL matrix. These results suggest the existence of non-specific binding to the base matrix. As mentioned in the introduction, the PBA ligand can participate in several secondary interactions with target proteins. Without secondary interactions, the non-glycosylated protein (RNase A) should not interact with the PBA resin and the glycosylated RNase B should bind solely through the esterification of the *cis*-diol groups present in its carbohydrate chain. The esterification reaction equilibrium is considerably higher at alkaline pH (pH >8) explaining the onset of adsorption observed in Figure 2 for the Agarose P6XL. The earlier onset observed for the ProSep®-PB media is probably due to electrostatic interactions with surface silanol groups on the silica support which have pKa values ranging from 4.9 to 8.5, depending on their microenvironment [33]. In addition, it is known that BA is in equilibrium with the hydroxyboronate anion and that the anion is also favored at alkaline pH values. Thus, PBA can also act as a weak cation exchange ligand at high pH, which may explain why the positively charged RNase A binds despite lacking a carbohydrate chain. In fact, the onset of RNase A adsorption is consistent with the pKas of the charged moieties on the resin, namely the matrix for ProSep®-PB and the ligand on the Agarose P6XL resin. To confirm these hypotheses, adsorption experiments were performed in the presence of 200 mM NaCl in order to mitigate the proposed electrostatic interactions (the affinity *cis*-diol interaction has been shown to be NaCl independent [9,16]). This strategy has been applied previously to prevent non-specific interactions in the purification of recombinant

antibodies [16]. As observed in Figure 2, only 10% of the non-glycosylated RNase A was observed to bind to the resin, while the adsorption of RNase B was minimally affected by the presence of NaCl. Further, adsorption of the non-glycosylated protein (RNase A) to bare CPG shows that the protein can adsorb to charged silanol groups on the CPG matrix. Together, this shows that electrostatic interactions can play a significant role in the adsorption of these positively charged proteins to the phenylboronate resin.

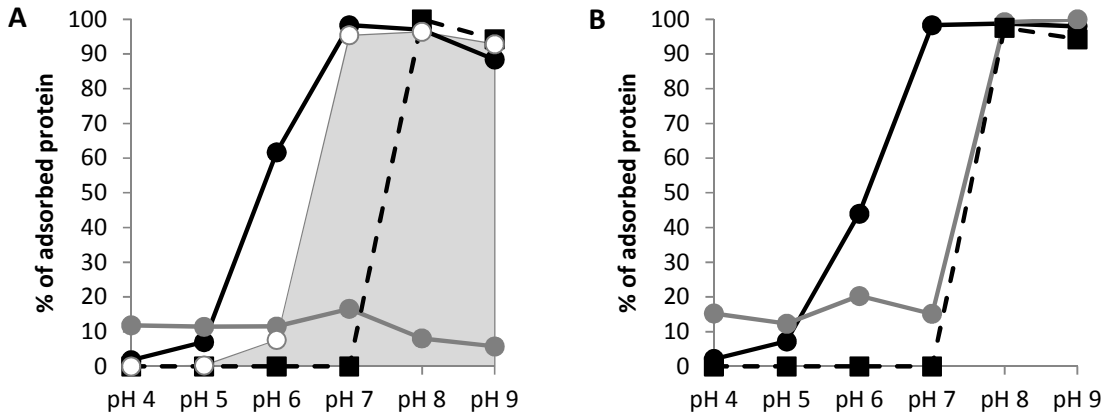


Fig. 2: Percentage of adsorption of basic proteins: (A) nonglycosylated RNase A and (B) glycosylated RNase B obtained in an Äkta Explorer system using different packed-bed columns: ProSep®-PB (straight lines, black circles ●), ProSep®-PB with 200 mM NaCl in adsorption buffer (straight lines, grey circles ●), Agarose P6XL (dash lines, black squares ■), and bare CPG® (full area, white circles ○).

3.1.2 Acidic proteins

For acidic proteins, adsorption decreased with an increase in the pH value (Figure 3). Furthermore, adsorption of the non-glycosylated pepsin (Figure 3A) persisted over a wider pH range (above 80% between pH 4 to 7), whereas adsorption of the glycosylated amyloglucosidase (Figure 3B) began to decrease around pH 6. The onset of

decreased adsorption on Agarose P6XL was earlier than on ProSep®-PB, analogous to the effect seen with basic proteins. Because adsorption occurs predominantly outside the pH range of *cis*-diol affinity interactions, protein adsorption must occur via non-specific interactions.

Adsorption of nonglycosylated pepsin to bare CPG was also measured but no interaction was observed. This was expected since negatively charged acidic proteins would be repelled from the negatively charged silanols (pKa 4.9-8.5) on the CPG matrix. This electrostatic repulsion can also explain the low binding of the glycosylated amyloglucosidase at high pH, which would be repelled by both the negatively charged silanols and the hydroxyboronate anion. To mitigate this repulsion, 200 mM NaCl was added to the adsorption buffers, resulting in the enhanced adsorption of both proteins. The adsorption of pepsin remained high up to pH 8, while the adsorption of amyloglucosidase decreased at neutral pH (pH 7) but increased abruptly for alkaline pH values. The behavior of amyloglucosidase was particularly interesting as it indicated that electrostatic repulsion was impeding the esterification of the glycan *cis*-diol groups at low ionic strength.

The stronger binding of pepsin under higher salt concentrations revealed that non-electrostatic secondary interactions are likely present at acidic and neutral pH values (which will be investigated in section 3.2).

A possible explanation for this observation arises from the fact that at these pH values, the BA ligand is a weak Lewis acid [14] with an incomplete valence that can interact with Lewis bases, such as carboxylic groups from aspartic and glutamic acid residues. In the hydroxylated tetrahedral form (boronate), it is no longer a Lewis acid and cannot participate in charge transfer interactions. Hence, binding of both proteins at acidic pH values is probably a consequence of these charge transfer effects which are absent at alkaline pH values, while binding of amyloglucosidase at alkaline pH values is triggered by esterification reactions but only if electrostatic repulsions are mitigated. The considerable

low adsorption of amyloglucosidase at pH 6 and 7 in the presence of NaCl suggests that binding of amyloglucosidase is salt dependent. According to Smith et al. [26], Lewis acids interact with Lewis bases via covalent or ionic bonds depending on the “hardness” of the interacting groups. This would explain why adsorption of amyloglucosidase was NaCl dependent but pepsin was not since hard Lewis bases tend to form ionic interactions meanwhile soft Lewis bases tend to form covalent bond [26]. In order to observe whether this was a general behavior for acidic glycosylated proteins, invertase was included as another acidic glycosylated protein in this study. It was observed that invertase did not bind at any pH value, except when NaCl was present in the binding buffer (to shield the electrostatic repulsion) and only for pH values above 7, where *cis*-diol interaction are more favorable. The high number of glycosylation sites of invertase (13 potential glycosylation sites according to supplier information) likely restricted access to the protein surface, hindering the formation of secondary interactions (charge transfer and also hydrophobic interactions) at lower pH values.

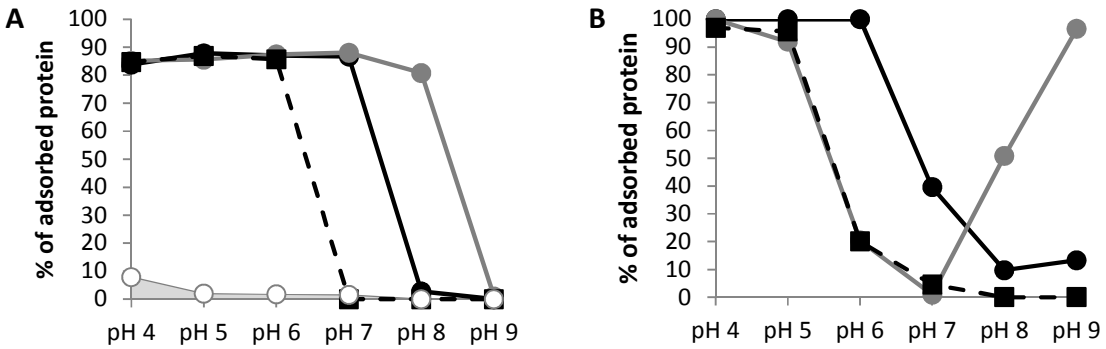


Fig. 3: Percentage of adsorption of acidic proteins: (A) nonglycosylated pepsin and (B) glycosylated amyloglycosidase obtained in an Äkta Explorer system using different packed-bed columns: ProSep®-PB (straight lines, black circles ●), ProSep®-PB with 200 mM NaCl in adsorption buffer (straight lines, grey circles ●), Agarose P6XL (dash lines, black squares ■), and bare CPG® (full area, white circles ○).

3.1.3 Neutral Proteins

As shown in Figure 4, neutral proteins (carbonic anhydrase, cellulase) were adsorbed over most of the pH range (pH < 9). Adsorption of the non-glycosylated carbonic anhydrase to ProSep®-PB was very strong (above 90%) between pH 5 and 8, even in the presence of 200 mM NaCl. Protein adsorption to bare CPG at lower pH (below the silanol pKas of 4.9 and 8.5) suggests that binding at low pH is not governed by electrostatic interactions. The predominant interaction is most likely charge transfer interactions as observed for the acidic proteins (e.g. pepsin). For the glycosylated cellulase, high adsorption to ProSep®-PB was persistent up to pH 8 and extended to pH 9 in the presence of 200 mM NaCl, which mitigated the electrostatic repulsion between the protein (pI 7.7) and boronate anion and allowed esterification to occur. The lower pH range of adsorption observed for the agarose matrix is probably due to steric hindrance as discussed in the next section 3.1.4. Hydrophobic interactions may also play a role in the adsorption of these proteins, however carbonic anhydrase has showed stronger evidence for this since adsorption to hydrophobic surfaces tends to increase near the protein pI (6.6), which is in the center of the pH range of this study.

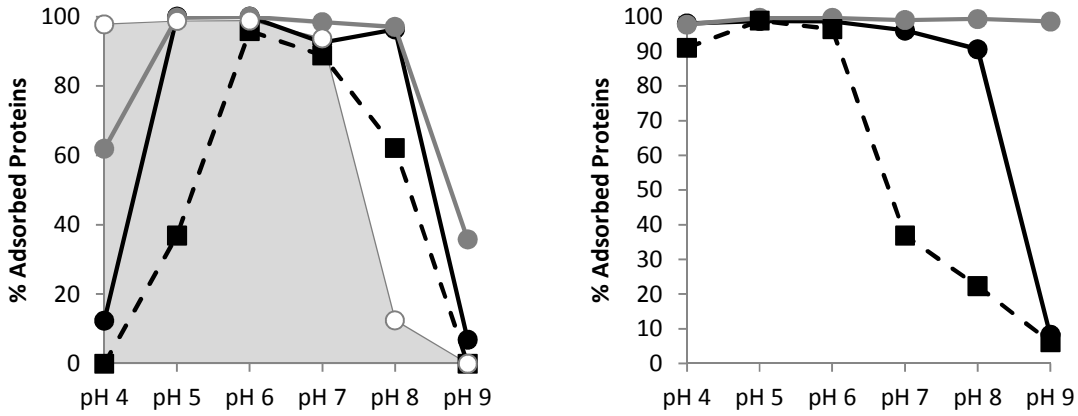


Fig. 4: Percentage of adsorption of neutral proteins: (A) nonglycosylated carbonic anhydrase and (B) glycosylated cellulase obtained in an Äkta Explorer system using different packed-bed columns: ProSep®-PB (straight lines, black circles ●), ProSep®-PB with 200 mM NaCl in adsorption buffer (straight lines, grey circles ●), Agarose P6XL (dash lines, black squares ■), and bare CPG® (full area, white circles○).

3.1.4 CPG versus agarose media

This library study showed that proteins adsorbed over a wider range of pH values on the ProSep®-PB than on the agarose P6XL matrix (Figures 2-4). In addition to the contributions of electrostatic interactions referred to previously, lowered steric hindrance and contribution of electrostatic interactions from negatively charged silanol groups could be responsible for extending the pH adsorption range on the CPG matrix. This matrix consists of larger and more controlled pore size (1000 Å) than P6XL and although studies using ARS dye, (section 2.3) showed that Agarose P6XL has a higher ligand density (14.26 ± 0.47 µmol/mL) than ProSep®-PB (8.76 ± 1.83 µmol/mL), the larger porosity of CPG matrices could increase the number of available ligands accessible to larger solutes such as proteins.

3.2 Batch adsorption studies: exploiting interactions at lower pH

In order to understand the importance of particular secondary interactions on protein adsorption in the lower pH rang (neutral trigonal conformation of PBA), the behavior of three proteins were investigated at pH 4 and 6 using batch adsorption experiments. The three proteins selected were the acidic nonglycosylated pepsin, the neutral nonglycosylated carbonic anhydrase and the neutral glycosylated cellulase since they exhibited a similar adsorption profile in the column studies. By selectively mitigating charge transfer interactions by the addition of Lewis base buffers (acetate, phosphate, fluoride and glycine) and/or hydrophobic interactions by adding ethanol, the importance of each interaction to protein adsorption could be evaluated. Table 1 (section 2.2.2) shows the expected effects of these mobile phase modifiers on protein-PB interactions.

The first modifiers studied were acetate (pH 4) and phosphate (pH 6) with concentrations ranging from 10 to 200 mM. Based on the peak adsorption of these proteins, pH 4 was selected for pepsin and pH 6 was chosen for the neutral proteins carbonic anhydrase and cellulase. As observed in Figure 5, neither acetate nor phosphate affected the adsorption of the selected proteins.

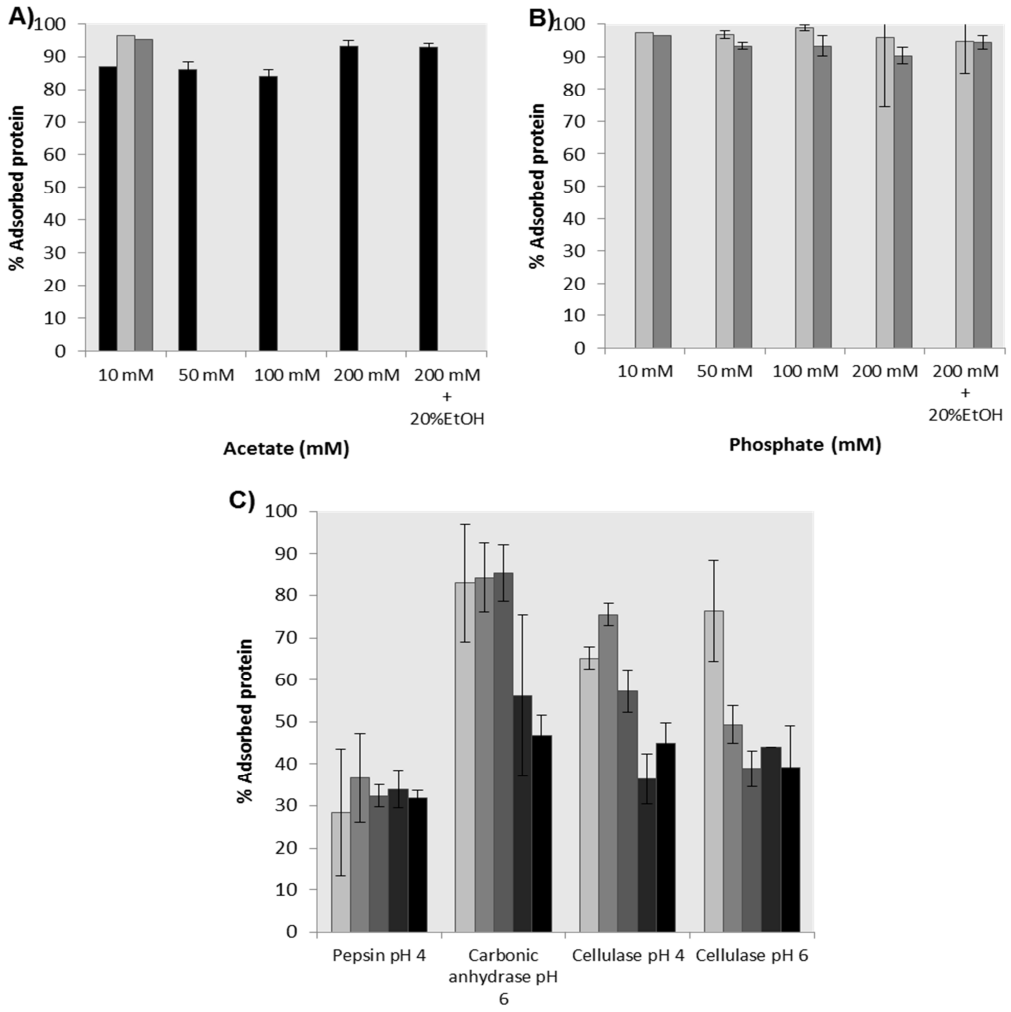


Fig. 5: Effect of (A) acetate and (B) phosphate in the percentage of adsorption of (■) pepsin, acidic nonglycosylated protein, at pH 4; (■) carbonic anhydrase, neutral nonglycosylated protein, at pH 6; and (■) cellulase, neutral glycosylated protein, at pH 6; obtained in batch adsorption experiments. Figure C represents the percentage of adsorption of pepsin, acidic nonglycosylated protein, at pH 4; carbonic anhydrase, neutral nonglycosylated protein, at pH 6; and cellulase, neutral glycosylated protein, at pH 4 and 6; obtained in batch adsorption experiments in the presence of different concentration of citrate: (■) 10 mM, (■) 50 mM, (■) 100 mM, (■) 200 mM and (■) 200 mM plus 20% ethanol.

Other Lewis bases selected were fluoride at 50 mM and glycine at 20 and 100 mM, neither of which had any effect on pepsin at pH 4. Adsorption of carbonic anhydrase at pH 6 was decreased by 15% using glycine at both concentrations. Cellulase at pH 6 was most sensitive to these Lewis bases, with a 35% decrease in adsorption using fluoride and 15% decrease by either 20 or 100 mM glycine. Due the toxicity of fluoride at higher concentrations, this investigation was stopped at an upper limit of 50 mM.

The next modifier studied was citrate, which in addition to being a Lewis base is also an α-hydroxyacid, containing three carboxylic acid groups in its structure. The effect is expected to be proportional to both the "hardness" and concentration of the Lewis base reagents [25,26]. Charged carboxylic acids are stronger Lewis bases than uncharged groups [26], thus citrate (containing three carboxylic acids) was tested at both pH 4 and 6 and is expected to be a "harder" Lewis base at pH 6. Citrate strongly influenced the binding of selected proteins (Figure 5C). Pepsin was strongly affected by citrate, retaining only 40% of the protein at concentrations of citrate as low as 10 mM. Cellulase adsorption decreased gradually with an increase in citrate concentration. At 200 mM citrate, only 40% of the protein was adsorbed to the resin. The adsorption of carbonic anhydrase was less sensitive to citrate, dropping only at 200 mM still retaining 55% of the loaded protein.

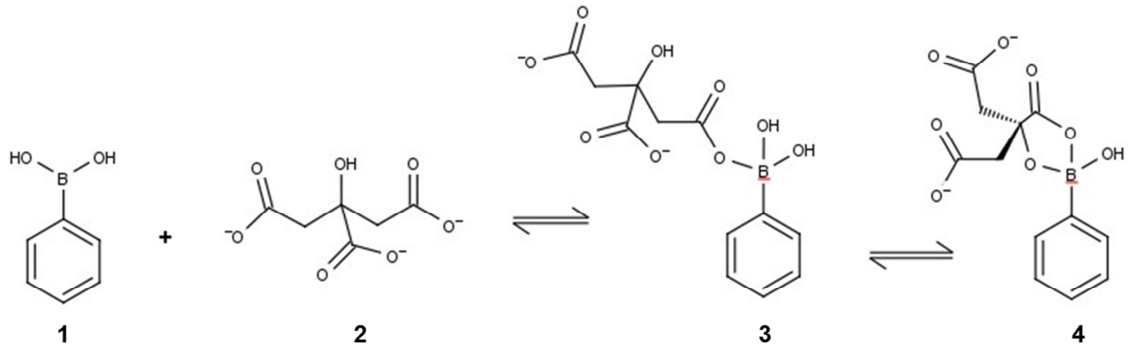


Fig. 6: Chemical equation of reaction between PBA (1) and citrate (2), an intermediate product formation (3) and their final cyclic complex formation as final product (4) [24].

According to Bosh et al. [24], the stepwise equilibria constant for the addition of different Lewis bases to phenylboronic acid increases in the order dihydrogenphosphate < hydrogenphosphate < citrate < hydrogencitrate < dihydrogencitrate, implying that the stability of phosphate complexes increases with deprotonation (and increasing negative charge) while the opposite is observed with the citrate complexes which are more stable with less charge. This idiosyncratic behavior is probably due to the fact that citrate can form a stable cyclic complex (Figure 6) with the boronic acid. Moreover, as citrate is a α-hydroxycarboxylate, it can interact with the boronic acid through an esterification reaction as it was a polyhydroxyl (diol) compound. These two effects can explain the higher efficiency of this carboxylate to mitigate protein binding to the ProSep®-PB resin but not the others (acetate and glycine).

Phosphate has thus exhibited a low effect on protein binding as it was studied at pH 6, and considering the different pKas, the most abundant form present at pH 6 is dihydrogenphosphate, which according to the previous series is the weakest Lewis base. Citrate on the other hand is expected to have more impact at lower pH when the dihydrogencitrate form prevails, i.e. at pH 4, which can explain why pepsin binding, at pH 4, was mitigated even at citrate concentrations as low as 10mM and why higher concentrations were required to diminish the binding of carbonic anhydrase at pH 6. Nevertheless, the binding of cellulase that was studied under both pH conditions shows no conclusive trend.

Another possible explanation for the differing effect of citrate lies with the composition of each protein surface, namely the location of charged groups and hydrophobic patches. As discussed before, charged carboxylic acids (aspartate and glutamate residues) could form charge transfer interactions with the boron atom from PBA under these conditions [26]. According to Figure 7A, pepsin has the highest number of

accessible, negatively-charged residues (red surfaces), which would increase the number of charge transfer interactions formed, but also make it susceptible to strong Lewis bases (e.g. citrate). On the other hand, the majority of negatively-charged residues on the surface of carbonic anhydrase (Figure 7B) are surrounded by positively charged residues, which could impede interactions with the PB ligand. However, some negatively charged residues are found near hydrophobic patches, as indicated by arrows in the Figure 7B. Here, charge transfer interactions with the boron atom may be stabilized by additional hydrophobic interactions with the phenyl group. Cellulase behaved similarly to pepsin, where citrate was effective at concentrations >50 mM (Figure 5C). As with pepsin, the negative charge patches on cellulase (Figure 7C – arrows at EP maps) are near hydrophilic patches, thus the charge transfer interactions are more susceptible to disruption from the hydrophilic citrate molecule.

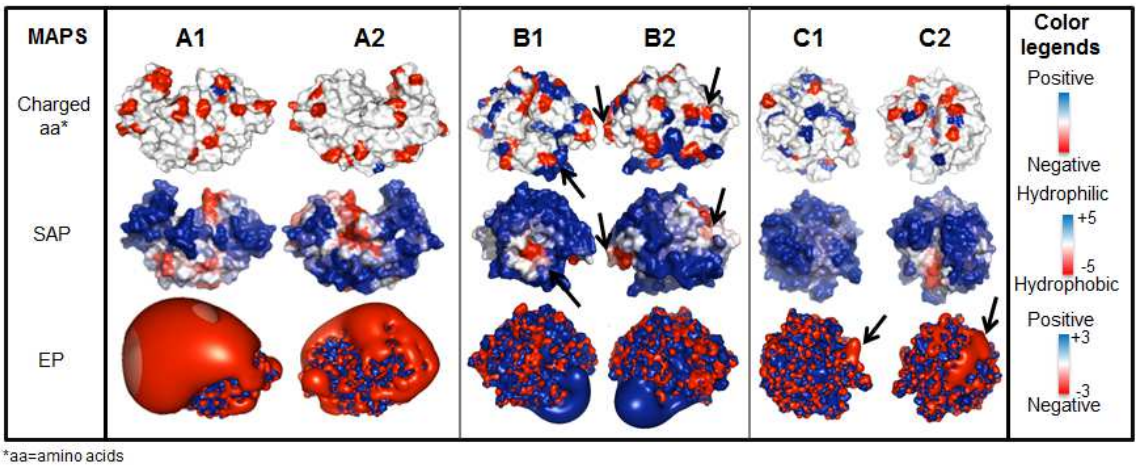


Fig. 7: Surface properties of pepsin (A), carbonic anhydrase (B) and cellulase (C) represented by charged amino acids (aa), spatial aggregation propensity (SAP) and electrostatic potential (EP) maps. Both front (1) and back (2) surfaces are represented by a 180º rotation. Arrows on B1 and B2 indicate the overlap between negatively charged aa and hydrophobic patches on charged aa and SAP maps, respectively. Arrows on C1 and C2 indicate the negative regions of cellulase represented in its EP map.

3.3 Evaluation of hydrophobic interactions

According to batch experiments results, a slight decrease of 25 % in adsorption was observed when 20% of ethanol was present in the adsorption buffer (data not shown) but when added in the presence of Lewis bases, namely acetate, phosphate and citrate at 200 mM, ethanol showed no additional effect (Figures 5A, 5B and 5C, respectively).

Although the above experiments were performed under low ionic strength conditions which are not favorable for inducing hydrophobic interactions, hydrophobic patches on the protein could be associating with the phenyl moiety of PBA in the background of stronger interactions (i.e. charge transfer). Within this context, two studies were performed to measure the hydrophobic potential of these proteins at low pH, using Phenyl HP Sepharose chromatography and the ANS fluorescence assay.

3.3.1 Phenyl HP Sepharose chromatography

The phenyl moiety of PBA could interact by hydrophobic or aromatic specific interactions (π-π or cation-π interactions) with protein surface groups. In order to measure the potential of the phenyl group to interact with the proteins in our library, we measured the isocratic retention of these proteins on a Phenyl HP Sepharose column. The retention times for each protein are summarized in Table 3.

Table 3: Retention time of Hi Trap™ Phenyl HP chromatography (1 mL of bed volume) under the same conditions as phenylboronate chromatography at pH 4, 6 and 9 with same buffers and pH 4 with 10 or 200 mM citrate. Flow rate was set at 1 ml/min. The column was washed with 20% ethanol between runs.

Proteins	Retention Time (min)				
pH	**4**	**6**	**9**	**4**	**4**
Buffer	50 mM Acetate	50 mM Acetate	20 mM CHES	10 mM Citrate	200 mM Citrate
Pepsin	1.3[T]	1.0	0.8	1.3[T]	1.1(23.2) 7.7(23.6) 12.3(53.2)[3]
Amyloglucosidase	0.9	0.9	0.8	-	-
Carbonic Anhydrase	1.5	1.1	0.9	1.1	1.1
Cellulase	1.6[T]	1.6[T]	0.8	1.5[T]	2.6
RNase A	2.5	1.1	3.3	1.9	1.0
RNase B	1.9	1.1	2.4	1.5	1.1

T=Long tail after peak; 3=number of peaks that was obtained in the chromatogram and the retention time corresponding to each peak. The values inside parenthesis are the percentage of peak area from respective retention time (mean values from duplicated experiments).

Initial experiments were performed under the same conditions used in the column experiments. It was observed that the pH had some influence in the retention of all proteins, with the exception of amyloglucosidase. Most proteins were eluted between 0.8

to 1.1 minutes, and tended to be retained longer at pH 4. Considering that the column volume is 1 mL, proteins with retentions time below 1.1 min probably were not retained and eluted right after the column dead volume. The basic proteins, RNase A and RNase B, had moderate interactions with phenyl sepharose at pH 4 and also at pH 9. The higher retention for these proteins could be explained by the relatively lower molecular weight compared to the other proteins (around 14 KDa according to supplier), in addition, no retention was obtained with these proteins on PB chromatography (Figure 2) under this condition. On the other hand, the retention was not observed at pH 6 for both RNase A and B, and at pH 9, the nearness of these proteins to their pI (Table 2) could be a reason for enhancement of their hydrophobicity. At pH 4, pepsin and cellulase had mild interactions, eluting early, but with a long tailing peak. Cellulase likely interacted because it is net positively charged at this pH, suggesting that cation-π aromatic interactions could be significant. Pepsin is net negatively charged under this condition, but the mild interaction could be attributed to hydrophobic surface patches, as observed at Figures 7A1 and 7A2.

The hydrophobic association in the presence of either 10 or 200 mM citrate, pH 4 was also measured in order to investigate whether citrate's effect is related to the phenyl moiety of PBA. According to results presented in the Table 3, citrate at low concentrations (10 mM) caused a slight decrease in the retention time of carbonic anhydrase, RNase A and RNase B, and did not affect retention of cellulase and pepsin, proteins which had higher sensitivity to citrate. On the other hand, 200 mM citrate enhanced the hydrophobic interactions for pepsin and cellulase that eluted in the form of three split peaks, in which the last only came out in the regeneration step performed with 30 mM acetate, pH 3. The higher concentration of this buffer probably increased salting out effects due to the higher concentration of ions present in the solution which induced hydrophobic interactions. In addition, the ability of this kosmotropic salt to promote hydrophobic associations also can

promote the aggregation of pepsin which might be responsible for the 3 peaks observed when 200 mM citrate was used.

Indeed, results from SEC showed that the second and third peaks were the ones where presence of aggregation occurred with a molecular weight (MW) 7.5-fold higher than the MW of pepsin although they were present in low amount (1.7% in the second peak and 14% in the third peak). Due presence of short secondary peaks at RNase A and B chromatogram of Phenylsepharose chromatography at pH 9 and cellulase at 200 mM citrate pH 4, they were also investigated by SEC and results showed that the MWs of these peaks did not correspond to RNase A, RNase B and cellulase MWs and they are probably impurities present in the commercial proteins used (Appendix A2).

3.3.2 ANS fluorescence

In order to characterize the protein surface in terms of hydrophobicity, ANS fluorescence was used since this technique has been reported as a probe for non-polar sites on proteins [34,35]. Studies were performed under the following binding conditions: pH 4, 6 and 9, with and without 200 mM NaCl and the results are presented in Figure 8.

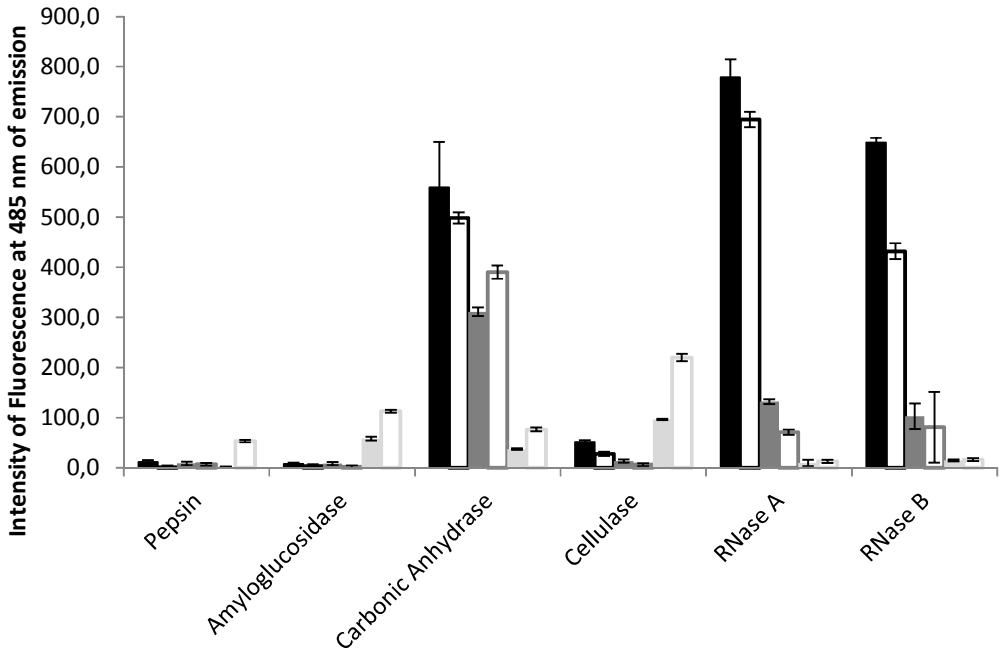


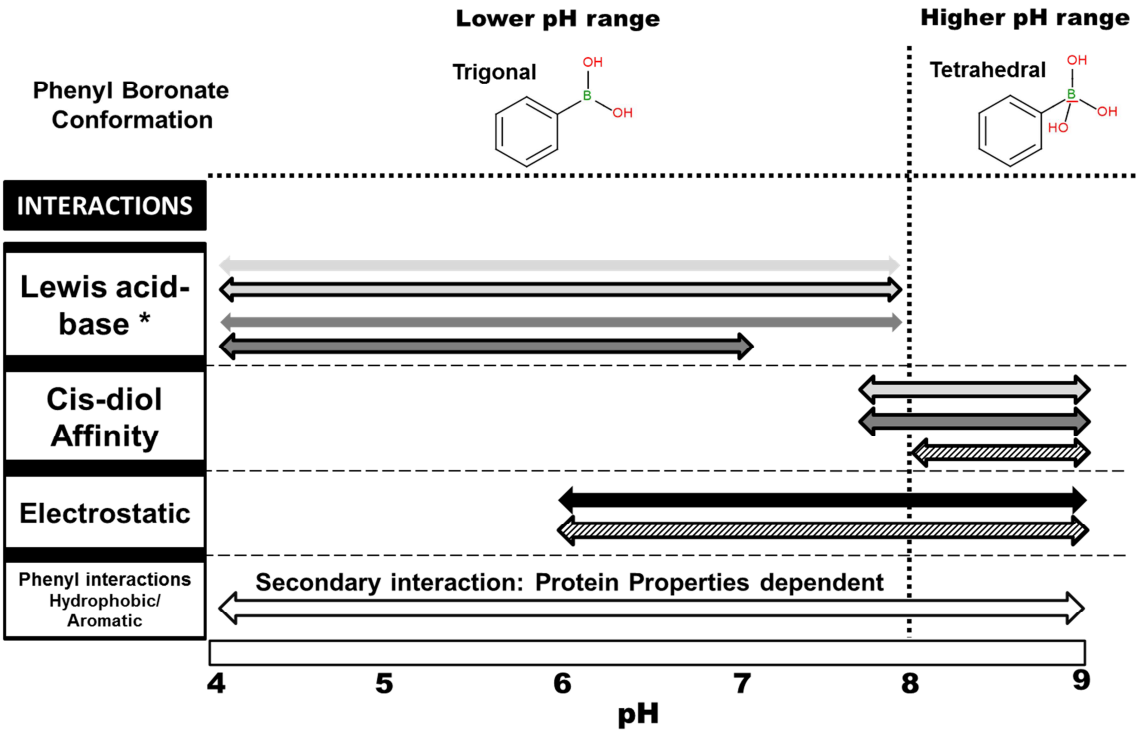
Fig. 8: ANS fluorescence of protein library at 485 nm (emission). Full bars: black - 50 mM Acetate, pH 4, dark grey - 50 mM Acetate pH6, light grey - 20 mM CHES pH 9. Open bars with respective colors represent same conditions (pH and buffer) supplemented with 200 mM NaCl.

Comparing results from Figure 7 and Figure 8, carbonic anhydrase, RNase A and RNase B at pH 4 are the only proteins for which the number of hydrophobic patches on the protein surface could be high enough in order to strengthen hydrophobic interactions. Cardomone et al. [35] have studied RNase A and B at alkaline pH using the same ANS method and found out that these proteins have a very hydrophilic surface which is consistent with results obtained at pH 9 for which a very low fluorescence was measured (Figure 8). In addition, it has been suggested that electrostatic interaction can increase ANS fluorescence at low pH. In fact, the anion ANS has been reported to bind to proteins via both hydrophobic and electrostatic interactions, especially at low pH when positively charged molecules are more abundant [36,37] . This effect seems to be observed in the

results of Figure 8; since all positively charged proteins at pH 4 and 6 (except cellulase) exhibited a high fluorescence emission when at a pH below their pI; the presence of 200 mM NaCl caused a decrease in this value and also an increase in the fluorescence of negatively charged proteins at pH 9, probably shielding the electrostatic repulsion with the now largely negative protein surface. Analyzing only the results at pH 9 (light grey bars), where electrostatic interaction is not present: cellulase, amyloglucosidase, carbonic anhydrase and pepsin had a relative high fluorescence emission which is in agreement with proteins that had higher adsorption on PB chromatography at lower pH conditions.

4. Conclusions

This work has shown that the adsorption of proteins to PB is highly dependent on several modes of interaction that are modulated by the pH and ionic strength of the medium, justifying its classification as a mixed-mode ligand (Figure 9). The adsorption trends were similar for Agarose P6XL and ProSep®-PB matrices for proteins within the same class (either acidic, neutral, basic, nonglycosylated or glycosylated), although an extended adsorption pH range was observed for ProSep®-PB. The narrower range of adsorption observed with Agarose P6XL could heighten the selectivity of PB chromatography through the elimination of protein interactions with the CPG base matrix.



* Interaction assumed by effect of citrate on adsorption.

Fig. 9: Map of protein adsorption on phenylboronate chromatography based on studies with protein library of this work. The y-axis represents the interaction responsible for adsorption in the specific pH range (x-axis). The pH range is divided in two groups, lower pH range with phenylboronic acid in a trigonal conformation and higher pH range when the ligand assumes a tetrahedral conformation. Light grey arrows are the ranges of acidic proteins, darker grey from neutral proteins and black from basic proteins. The black straight lines are the range of glycosylated proteins from the classes with respective colors and the one with dashed arrows are the glycosylated basic proteins. The white arrow corresponds to phenyl interactions which are dependent on protein surface properties as presence of hydrophobic patches.

The operating ranges of these different interactions have been determined from the various adsorption experiments performed in this study. 200 mM NaCl shielded all the

electrostatic interactions (attractive and repulsive) between proteins and PBA, mitigating non-specific adsorption of basic proteins and allowing negatively-charged glycosylated proteins to approach the surface and bind to PBA via *cis*-diol interactions. *Cis*-diol affinity interactions were found predominantly at higher pH values and were shown to be NaCl independent. The presence of charge transfer interactions had been demonstrated by the competition effect of citrate, a strong Lewis base, on the adsorption of selected proteins at low pH (pH 4 and 6). This Lewis base buffer was more effective at competing with proteins that interacted primarily via charge transfer interactions and was reduced in the presence of secondary hydrophobic interactions.

Since adsorption still occurs without *cis*-diol affinity, electrostatic, charge transfer interactions, and hydrophobic or aromatic interactions with the phenyl moiety are likely present during protein adsorption. Hydrophobic/aromatic interactions can occur at all pH conditions (Figure 9) although the conditions studied are not favorable for inducing them.

This work has provided further insight into the mixed mode interactions of proteins with PB resin systems. The work has identified two distinct pH ranges in which phenylboronate have very different interactions with proteins. In addition to the highly exploited *cis*-diol affinity interaction that is dominant at alkaline pH, charge transfer interactions can be exploited for the separation of biomolecules at lower pH. By rationally exploiting these interactions, highly selective windows of separation could potentially be achieved for the fractionation of complex biological mixtures, opening up new uses of this ligand in the manufacturing of biopharmaceuticals.

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CHAPTER IV

EXPLORING THE SELECTIVITY OF PHENYLBORONATE CHROMATOGRAPHY: PROOF OF CONCEPT

To Submit

Results presented in the following conferences:

Carvalho, R.J., Woo, J., Parimal, S., Aires-Barros, M.R., Cramer, S.M., Azevedo, A.M. Phenylboronic acid as ligand for multimodal chromatography: proof of concept – Affinity, Vienna/Austria, 2013.

Carvalho, R.J., Woo, J., Nakamura, K.A., Aires-Barros, M.R., Azevedo, A.M., Cramer, S.M. Phenylboronic acid as a multimodal chromatography: adsorption behavior comparison between control pore glass and agarose matrices – 33rd ISPPP, Boston/USA, 2013.

Exploring selectivity of phenylboronate chromatography: proof of concept

Abstract

Phenylboronate chromatography (PBC) can be a complex system due to its multimodal behavior but also provide higher selectivity and capacity. Herein, elution strategies were carried out in order to improve PBC selectivity. Gradients of reverse salt, pH and displacers were investigated. The best results of selectivity were obtained when the modifiers tris at pH 9 and citrate at pH 5 were applied in gradient elution. Tris selectively separated glycosylated proteins retained at alkaline pH by affinity *cis*-diol interaction. When an artificial mixture of the proteins was loaded, a gradient rate of 0.57% tris/min provided selectivity separation. Gradient elution was also performed for acidic and neutral proteins separation. These proteins were selectively eluted when citrate gradient was applied at acidic pH and the same rate of 0.57% citrate/min provided the best peak separations. Gradients strategies by reverse salt and pH were not able to elute completely the proteins during the process. The repulsion by negative charges which avoid negatively charged proteins to bind at PBC at pH 9 is not strong enough to elute these proteins once they were retained suggesting the hypothesis that desorption mechanism of PBA behaves differently from adsorption. The pH gradient results can reinforce this hypothesis since the proteins were not totally recovered during the gradient what was not expected for most of proteins applied. This work provided not only improvements on PBC selectivity but also important insights of this chromatography which can help understand its mechanisms of adsorption and desorption.

Keywords: Phenylboronate chromatography, selectivity, elution studies, proteins purification, glycosylation

1. Introduction

Phenylboronate chromatography is known by a unique interaction able to reversible esterify with *cis*-diol content molecules classifying it as affinity chromatography [1]. For more than 40 years this chromatography has been studied and applied in separation of *cis*-diol biomolecules including carbohydrates [2], nucleosides [3], ribonucleic acid (RNA) [4,5] and glycoproteins [6-8]. This wide range of possible applications make this affinity chromatography a powerful separation technique of *cis*-diol biomolecules. However, mechanisms of phenylboronate interaction with these specific molecules are not completely understood since several external variables can affect it [9]. It is already known that under alkaline conditions PBA changes from trigonal to tetrahedral conformation due to free p orbital of boron able to coordinate Lewis bases molecules as hydroxyls [10]. According to the literature, tetrahedral conformation enhances the *cis*-diol affinity interaction providing higher association constants [11]. In addition, 1,2-, 1,3- or 1,4 *cis*-diol can esterify with boronic acids in order to form 5, 6 or 7 boronate ester, respectively and the geometry of 1,2 *cis*-diol has been reported as the most favorable for esterification [12]. Moreover, radical substitute of diol compounds can also influence in this interaction [13]. For instance, glucose and sorbitol, saccharides with similar structures obtain different values for association constants at same conditions, 11 and 1000 M^{-1} (both at pH 8.5), respectively, and catechol, an aromatic ring with 1,2 *cis*-diol in the ring surface reaches 3300 M^{-1} at same pH [11]. These variables come up with pH dependency of phenylboronic acid due to the conformation changing but also with a very specific interaction [9].

The miscomprehension of some results obtained by PBC has being currently directed to the multimodal behavior of this chromatography, i.e., the ability to perform different interactions (Figure 2 – chapter II) [6]. Boronic acids are negatively charged at tetrahedral conformation due to the hydroxylation at conditions with pH equal or above

PBA pKa (8.8), allowing this ligand to act also as cation exchanger [9]. Studies has been used NaCl in order to shield electrostatic interactions of impurities and increase selectivity of PBC [14], but also for avoiding electrostatic repulsion of the target biomolecule [15]. The phenyl moiety of PBA can also provide hydrophobicity or aromatic interactions as π-π or cation-π interaction [9]. Since low ionic strength is usually used in order to avoid induction of hydrophobicity, aromatic interactions may have higher probability to promote binding [7]. In addition, hydroxyls can also have some effect on interaction by hydrogen bond although is not strong enough to capture large biomolecules, even though this interaction has been explored in this chromatography for capture of serine proteinases [16]. According to the literature, the multimodal chromatography (MMC) brings several advantages over single mode chromatography like better selectivity and higher capacities [17]. Capto adhere, the N-benzyl-N-methyl ethanol amine ligand, is an example of multimodal interaction performance. This anion-exchange chromatography with hydrophobic moiety has selectively separated single from double-stranded DNA by simple linear salt gradient [18] demonstrating the high selectivity of MMC. Facing these important features, this type of chromatography became a current strategy for obtaining more efficient purification process than the traditional single mode chromatography [17]. However, MMC is also a complex system and deeper studies on mechanisms of these interactions are needed. PBC is a clear example of this complexity since several studies have demonstrated nonspecific interaction as the drive interaction between protein and PBA [15].

PBC is usually performed for capturing of diol containing molecules, especially glycosylated proteins [8,9,15]. The usual binding condition for the separation process of this class of protein is operated at alkaline condition in order to retain specifically glycosylated by tetrahedral conformation of PBA [9,14]. Efforts in obtain lower binding pH closer to neutral has been made by replacing the substitute radical of PBA, however none of them were stable or efficient as PBA [19]. Studies also have been shown a possible

retention of glycosylated proteins at neutral and acidic conditions using PBA as ligand [14]. These results have also been observed at adsorption studies at chapter III where secondary interactions played an important role for retention of proteins, especially at conditions when PBA is mostly in trigonal conformation as neutral pHs.

The most common elution strategies used for eluting diol compounds from PBC are either displacer gradient using *cis*-diol compounds or pH dropping [14,20]. Saccharides as sorbitol, fructose and mannose can be often found as displacers although a further step for eluting these saccharides is necessary in order to regenerate the column [10]. Tris(hydroxymethyl)aminomethane (tris) has also been applied as eluent in PBC, although it has been reported as shielding agent, tris has efficiently recovered polyclonal human IgG from PBC [14]. Dropping the pH to acidic conditions (pH 2-3) is another strategy for recovering diol compounds from PBC. According to the literature, the lower the pH, the lower is the association constant between *cis*-diol and PBA [11,13]. Although this strategy is usually used, the elution conditions can be aggressive to the protein structure. In addition, low recovery efficiency was observed for proteins [14] but high efficiency has been obtained for molecules as nucleosides.

Elution strategies are performed not only for developing a process but also for fundamental studies on protein-ligand interactions in order to obtain a better comprehension of such mechanisms [21]. Strategies vary according to the chromatography used [22]. Affinity chromatography usually requires the presence of displacer with same interaction as the target molecule in the elution buffer [1]. Same elution strategies for MMC can enhance its performance as applying a multimodal elution using different modifiers and conditions. Holstein et al. [17], using a protein library, applied several modifiers used in single mode chromatography in order to obtain higher selectivity of a multimodal cation exchange chromatography (Capto MMC). Among them, arginine and guanidine were found to improve the selectivity of Capto MMC by their ability to

stabilize proteins structures [23,24]. In this study, pH gradient was investigated due the pH dependency of this chromatography. The concept of multimodal elution was also studied by applying the following modifiers in the gradients performed: tris, citrate, boric acid and urea. The selection of modifiers for this work is related to their effect on boronic acids reported in the literature. For instance, tris has showed ability to interact with PBA by tridentate interaction at high pH when applied as shielding reagent [25] and elution buffer [14,26]. Borate has been also reported of being able to interact with boronic acids by their removal from contaminated water [27]. Although pH ≥ 11 was necessary in order to obtain good performance of removal, a study using borate in the washing buffer for capturing and washing glycosylated proteins from anion exchange chromatography successfully removed host glycosylated protein from *Pichia pastoris* at pH 8 [28]. Citrate is a strong Lewis base compound with three carboxylic acid moieties and also an α-hydroxyacid which enables this acid to perform either charge-transfer or a chelate interaction with trigonal conformation of PBA [13,29]. The use of urea in chromatographic systems is widely discussed since it can behave as denaturing agent in at high concentration. Even though, studies have been used this agent for eluting biomolecules from PBC bound by nonspecific interaction [15] and hydrogen bond [30].

In this work, we set different elution strategies for enhancement of PBC selectivity. A protein library based on presence of glycation and pI values were used in order to evaluate the influence of each elution. The following elution strategies were carried out: reverse gradient at alkaline pH in an attempt to separate acidic and neutral glycosylated proteins by charge repulsion; pH gradient in a crescent and descent gradient values from a pH range 4 to 9 for separating acidic/neutral proteins and glycosylated proteins, respectively; and finally, displacer gradient using the modifiers aforementioned. This study not only provides selective ways for PBC elution but also important insights on its multimodal behavior.

2. Materials and Methods

2.1 Materials

The protein library was composed by the following proteins obtained from Sigma-Aldrich® (St. Louis, MO, USA): amyloglucosidase from *Aspergillus niger*, carbonic anhydrase from bovine erythrocyte, cellulase from *Trichoderma reesei*, conalbumin from chicken egg white, invertase glycoprotein standard from *Saccharomyces cerevisiae*, pepsin from porcine stomach, ribonuclease A (RNase A) from bovine pancreas and ribonuclease B (RNase B) from bovine pancreas.

2-(Cyclohexylamino)ethanesulfonic acid (CHES), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS), 3-Morpholino-2-hydroxypropanesulfonic acid (MOPSO), 2-(4-morpholino)ethanesulfonic acid (MES), [(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid (TAPS), sodium phosphate mono and dibasic anhydrous, tri-sodium citrate anhydrous, sodium acetate anhydrous, tris(hydroxymethyl)aminomethane (Tris) and sodium chloride were also purchased from Sigma-Aldrich. Ethanol was purchased from Panreac (Panreac, Barcelona, Spain) and glacial acetic acid from VWR (VWR, Ecuador). The chromatographic media used in this study were aminophenylboronic acid functionalized CPG® (ProSep®-PB, EMD Millipore, UK), Agarose P6XL (ProMetic Biosciences, UK). All reagents used had a purity ≥ 98%, were PA or of HPLC grade. All the water used in the experiments was obtained from MilliQ purification system (EMD Millipore, USA).

Äkta Explorer and Purifier systems (GE Healthcare, Uppsala, Sweden) were used to perform all column experiments, both systems were operated online by the software Unicorn 5.11. Tricorn™ empty glass columns (i.d. 5 mm; max. bed height 109 mm) were used for packing ProSep®-PB and Agarose P6XL to a column volume of 1 mL (CV). Batch

experiments were performed with MultiScreen™-HV 0.45 µm Durapore 96-well membrane plates (EMD Millipore, USA).

2.2 Elution experiments in column

As a continuation of the adsorption studies, the proteins used as samples were basically the same used in the work of chapter III, they were divided in two main groups, glycosylated and nonglycosylated, and both groups, acidic, neutral and basic proteins were used (Table 2 – chapter III). They were dissolved in the respective binding buffer and separated loaded in the column in amount of 100 µg, except invertase with 17.5 µg. Low loadings were performed in order to avoid saturation of the column.

Different elution strategies were performed for selectivity studies of PBA chromatography. Different modifiers were used for eluting depending on the protein class used based on results obtained in the adsorption studies (chapter II) as well as the binding conditions for each class. The studies performed with glycosylated proteins were performed at alkaline binding condition (pH 9) for glycosylated proteins and acidic binding condition for acidic and neutral proteins (pH 5). The elution strategies are further described.

2.2.1 Reverse salt gradient

This study was performed at pH 9 using 50 mM CHES buffer supplemented with 300 mM NaCl and acidic (invertase, amyloglucosidase) and neutral (cellulase) glycosylated protein as samples and 1 mL/min of flow rate. ProSep®-PB packed in 1 mL glass column was used as stationary phase. The process used is a linear reverse gradient (from 300 mM to 0 mM of NaCl) with same buffer without any supplementation in the end. Cleaning step was performed with 1.5 M tris-HCl at pH 9, condition reported as able to completely elute proteins from PBC [14]. The Figure 1 shows schematically the process

performed. The percentage of protein was determined based on peak areas obtained in the chromatogram divided by the total peak area. The experiments were duplicated performed.

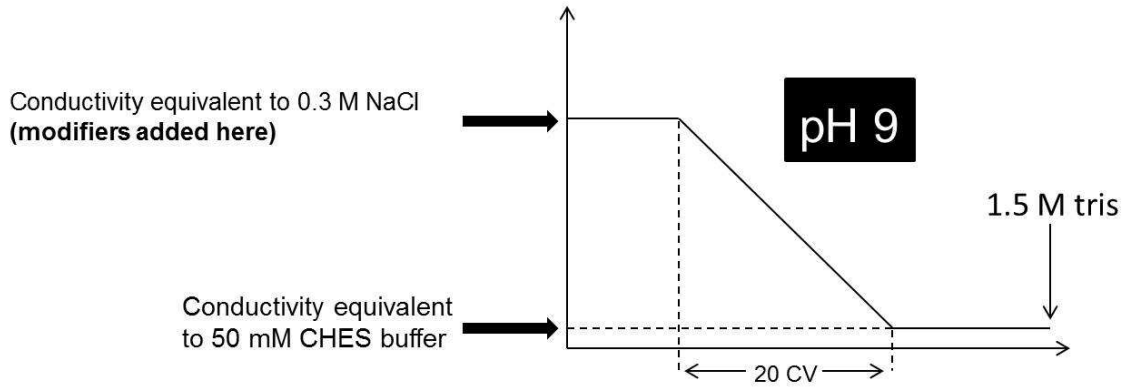

Fig. 1: Scheme of reverse gradient performed at pH 9 in liquid chromatography using 1 mL ProSep®-PB column.

The modifiers used were also based on adsorption studies (chapter III) or works in the literature [15,25,28]. Table 1 shows the buffers, salts and modifiers used in this study.

Table 1: Composition of binding and elution buffers at pH 9 used in the reverse gradient study.

Binding			Elution
Buffer	**Salt**	**Modifier**	**Buffer**
50 mM CHES	0.3 M NaCl	-	50 mM CHES
50 mM CHES	0.3 M NaCl	50 mM Citrate	50 mM CHES
50 mM CHES	0.3 M NaCl	50 mM Boric acid	50 mM CHES
50 mM CHES	0.3 M NaCl	2 M Urea	50 mM CHES
50 mM CHES	0.3 M NaCl	15 mM Tris-HCl	50 mM CHES
50 mM CHES	0.18 M $(NH_4)_2SO_4$	-	50 mM CHES
20 mM CHES	0.2 M $CaCl_2$	-	20 mM CHES
20 mM CHES	0.2 M $MgCl_2$	-	20 mM CHES
50 mM HEPES*	0.3 M NaCl	-	50 mM HEPES*

*pH 8.5 was used for HEPES buffer.

2.2.2 pH gradient

According to the chapter III, PBA is a pH dependent ligand. This dependency can be explored by using pH gradient. The studies of pH gradient using in this PBC work were based on Kröner and Hubbuch studies [31]. This work provided a systematic generation of buffers composition based on their pKa with linear titration curves in order to control the pH gradient of ion exchange chromatography. The buffer composition in this work is described in Table 2 and all the components are acidic buffers. Three buffer compositions were prepared, one for glycosylated protein with supplementation of 300 mM NaCl binding at pH 9 (BGly) and other two for acidic and neutral proteins with binding between pH 4 and pH 5 with and without one modification in their compositions (BAN1 and 2).

Table 2: Buffer compositions of pH gradient studies. Compositions of buffer for glycosylated protein (BGly), buffer for acidic and neutral proteins (BAN) 1 and 2.

Buffer components	Buffer composition: BGly	Buffer composition: BAN1	Buffer composition: BAN2
9.4 mM CHES	X	X	X
4.6 mM TAPS			X
4.6 mM HEPES	X	X	
9.9 mM EPPS	X	X	X
8.7 mM MOPSO	X	X	X
11 mM MES	X	X	X
13 mM Acetate	X	X	X
300 mM NaCl	X		

The buffers were weighted and dissolved in MilliQ water in a known volume. This volume was divided in two equal parts, one parte had the pH adjusted to 9 with 1 M NaOH and other to pH 4 or pH 5 using concentrated HCl. After pH titration, the volume of each buffer was completed with MilliQ water.

The process was performed as linear gradient of buffer B (buffer with final pH of the gradient) with 15 CV of gradient length starting with buffer A as binding condition, after 5 CV at 100 % of buffer B, a cleaning step was performed with 1.5 M tris-HCl. For glycosylated proteins binding buffer A was the BGly at pH 9 and buffer B BGly at pH 4 and for acidic/neutral binding proteins buffer A was BAN (1 or 2) at pH 4 or 5 and buffer B BAN (1 or 2) at pH 9. This range of pH was selected based on adsorption studies of previous chapter.

Samples from both groups of the protein library were used in the pH gradient studies separately loaded. The following glycosylated proteins were used: RNase B, cellulase, amyloglucosidase and invertase and as acidic/neutral proteins: amyloglucosidase, pepsin, cellulase, conalbumin and carbonic anhydrase.

Two stationary phases were used ProSep®-PB (controlled-pore glass) and P6XL (agarose) matrices packed in 1 mL columns in order to compare the effect of different matrix composition in this strategy. Runs were performed at 1 mL/min of flow rate. The percentage of protein was determined based on peak areas obtained in the chromatogram divided by the total peak area from duplicated experiments.

2.2.3 Displacer gradient

A similar process to pH gradient was performed: a linear gradient of 20 CV with 5 CV of plateau when the gradient reached 100% of buffer B. Cleaning step with 1.5 M tris-HCl was also carried out in these studies. As pH gradient processes, runs were performed at 1 mL/min of flow rate. The calculation of percentage of protein was also determined based on peak areas from duplicated experiments. 1 mL of ProSep®-PB packed was used as stationary phase.

The same samples from section 2.2.2 were used and divided in the same groups as glycosylated and acidic/neutral proteins. The binding conditions applied for glycosylated

proteins were 20 mM CHES pH 9 supplemented with 300 mM NaCl and the same buffer was used for preparing the elution buffers. Different chemical compounds able to interact competitively with PBA were applied as elution buffers: 0.5 M tris-HCl, 0.5 M D-sorbitol, 0.5 M Fructose, 0.5 M citrate, 0.5 M boric acid and 0.1 M lactic acid. For the second group of proteins 50 mM acetate pH 5 was used in the binding condition and used for preparing the following elution buffers: 0.5 M tris-HCl and 0.5 M citrate.

2.3 Adsorption batch experiments

The effect of citrate was investigated by batch experiments. The binding conditions were performed with the following buffers: 10, 50, 100 and 200 mM citrate at pH 4 and 50 mM acetate pH 4 and 20 mM CHES pH 9 were used as negative and positive control, respectively. Commercial RNase A was used as sample at 1 mg/mL dissolved in the respective binding buffer.

The adsorptions were performed in 96-well microplates with membranes, 200 µL of protein solution was added in 20 µL of ProSep®-PB and agitated for 5 h using shaker-incubator Stat Fax™ 2200 (Bio-Rad, USA) at maximum velocity and then the supernatant was vacuum into UV 96-well microplate and the values of samples at UV 280 nm was obtained with SPECTRAmax Plus 384 microplate reader (Molecular Devices, USA). The percentage of adsorption was calculated based on Eq. (1):

$$P = \frac{Ci - Cf}{Ci} \times 100 \quad (1)$$

where P is the percentage of protein adsorption, *Ci* the initial concentration of protein (mg/mL), *Cf* the concentration of supernatant obtained after adsorption (mg/mL). The experiments were performed in triplicate.

2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed for qualitative analysis of commercial protein samples and fractions obtained from liquid chromatographic separations using an artificial mixture of different commercial proteins. Proteins were obtained from a known concentration and diluted with loading buffer in order to obtain 5 µg of protein loaded in the gel. Samples from chromatographic fractions were previously concentrated around 13-fold by Amicon ultra with Ultracel® - 3K cellulosic membrane (Millipore, Ireland). The loadings were prepared with 4x Lemmli sample buffer (Bio-Rad laboratories, USA) in denaturing conditions with dithiothreitol and they were pre-heated at 100ºC for 5 min prior loading in the gel. They were applied in a 12% acrylamide gel, prepared from a 40% acrylamide/bis stock solution (29:1) from Bio-Rad (Hercules, CA, USA), and ran at 150 mV using a running buffer that contained 192 mM glycine, 2.5 mM Tris, and 0.1% SDS, pH 8.3. Gels were stained with Coomassie Brilliant Blue.

3. Results and Discussion

3.1 Reverse gradient

In the adsorption studies of chapter III the results of interaction by esterification of *cis*-diol from glycosylated proteins to PBA demonstrated to not be NaCl dependent until 200 mM NaCl since RNase B (basic glycosylated protein) kept binding at pH 9 in the presence of NaCl at binding condition. However acidic and neutral glycosylated proteins were retained only in the presence of NaCl by shielding electrostatic repulsion between negatively charged proteins and PBA. Thus, this strategy was carried out as an attempt to separate selectively negatively charged proteins by repulsion, recovering more

electronegative proteins first by decreasing salt concentration in a reverse gradient since basic proteins would not elute under this condition.

Invertase, amyloglucosidase (acidic glycosylated proteins) and cellulase (neutral glycosylated proteins) were separately loaded in the column with 300 mM of NaCl at binding condition and a decreasing of this salt to 0 mM was performed. The first results showed that all proteins were recovered around the same conductivity (1.5 mS/cm) obtained in the end of the gradient. According to the Figure 2 (binding condition 1), we can observe that all invertase retained was eluted in the gradient step whereas amyloglucosidase and cellulase were only fully recovered at cleaning step. In order to obtain higher selectivity, different binding conditions were applied by supplementation of binding buffer with different modifiers: citrate, boric acid, urea and tris. As discussed in the chapters II and III, citrate is able to interact by two forms, from Lewis acid base carboxylate moiety or chelating cyclic interaction (Figure 6 of chapter III) [13]. In addition, citrate is known as a kosmotrope salt [32] able to perform salting out and induce hydrophobic interactions, however 50 mM is not high enough to promote significantly these interactions. Only invertase showed stronger retention compared to the condition without this acid being partially recovered at cleaning. When 50 mM of boric acid was used, even lower effect was obtained, the profiles were similar compared to the condition without this acid although boric acid has been already described in the literature as able to wash out glycosylated protein bound at cation exchange chromatography in the same concentration [28]. Another attempt has been performed with 2 M urea since it is known as denaturing agent and it has been reported as capable to elute recombinant erythropoietin from boronate chromatography [33]. On the other hand, studies of nonspecific interactions between heavy chain of recombinant human antibody (nonglycosylated chain) required 6 M of this agent to obtain 100% recovering, but 2 M was enough to start affecting the nonspecific interaction by a small peak eluted [15]. In the same work, the authors did not recovery the

glycosylated light chain interacted with PBA by esterification in any concentration of urea ranging from 2 to 6 M. In this study, 2 M provided stronger binding of invertase compared to the first condition without modifier, but no effect on amyloglucosidase and invertase was obtained. Invertase was highly retained even after elution and 70% was recovered in the cleaning step. These results showed that the nonspecific interaction obtained by nonglycosylated IgG heavy chain is probably not the same for any of these three proteins.

Tris at low concentration (15 mM) was applied with same intention of shielding effect as Li et al. [25]. This condition had a strong effect on cellulase binding, more than 60% was obtained at flow through. Apparently, secondary interactions of cellulase could be shielded, but these interactions were not affected by urea or the other modifiers used in this study. On the other hand, 15 mM tris affected other proteins since higher recovering by electrostatic repulsion was obtained when the conductivity decreased. Comparing these modifiers, tris seems to provide a higher selectivity when this process is used, although any of these modifiers have not changed significantly the conductivity in the recovering.

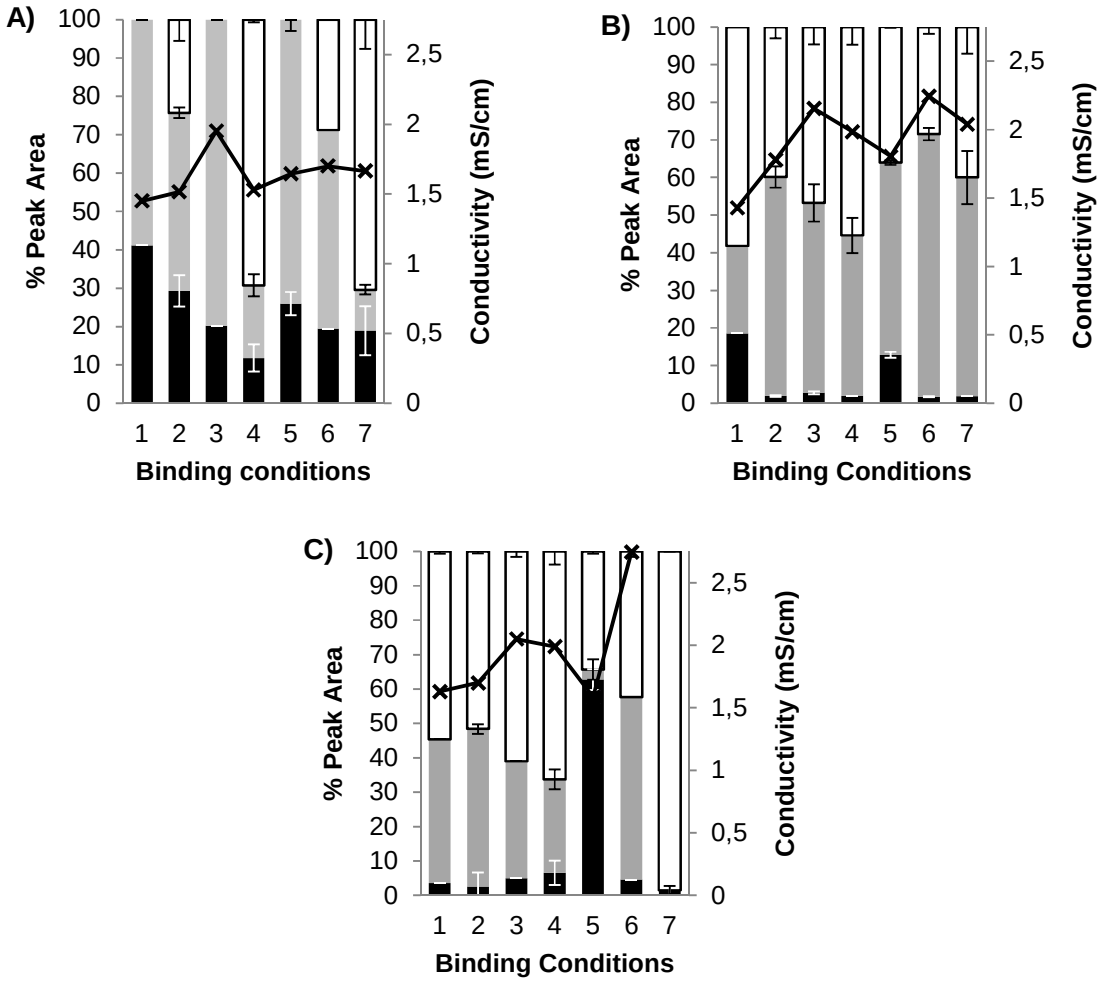

Fig. 2: Percentage of peak area of glycosylated proteins invertase (A), amyloglucosidase (B) and cellulase (C) at flowthrough (black fraction of the bars), reverse gradient (gray fraction of the bars) and cleaning step (white fraction of the bars) and conductivity of elution (x points) of PBC using reverse salt gradient process with different binding conditions. The binding conditions used were: 1 – CHES pH 9 + 0.3 M NaCl; 2 – Condition 1 supplemented with 50 mM citrate; 3 – Condition 1 supplemented with 50 mM boric acid; 4 – Condition 1 supplemented with 2 M Urea; 5 – Condition 1 supplemented with 15 mM tris; 6 – CHES pH 9 + 180 mM $(NH_4)_2SO_4$; 7 – HEPES pH 8.5 + 0.3 mM NaCl.

The salt effect was also studied by switching NaCl by $(NH_4)_2SO_4$, $CaCl_2$ or $MgCl_2$. As citrate, $(NH_4)_2SO_4$ is a kosmotrope salt usually used in hydrophobic chromatography, the concentration of 180 mM was used in order to obtain the same initial conductivity as 300 mM NaCl around 30 mS/cm. Although a small inducing of hydrophobicity was expected, the profile of proteins retention were similar, all three proteins were partially recovered in the end of the gradient. Regarding $CaCl_2$ and $MgCl_2$, chaotrope salts, the proteins were recovered only at cleaning step (data not shown) showing that these salts provided strengthen of *cis*-diol interactions compared to NaCl. Mg^{+2} has already been reported as enhancer the glycosylated proteins binding by previous studies [5,7,14], which could be related to cation-π interactions with phenyl moiety [7].

Different buffers have also been reported to affect interactions of PBA [11,13]. HEPES, as Mg^{+2}, is referred in the literature to enhance *cis*-diol affinity binding to PBA [13,14]. In fact, when this buffer was used at pH 8.5 all proteins had higher retention. Cellulase was all recovered at cleaning step whereas amyloglucosidase and invertase were partially eluted in the end of gradient (70% and 10%, respectively).

Although any condition has not effectively separated the proteins, both conditions: HEPES supplemented with NaCl and CHES supplemented with NaCl and tris provided the most favorable scenario between the conditions studied. It is also important to note the difference between the mechanisms of binding and elution. The electrostatic repulsion is strong enough to avoid binding of negatively charged proteins, on the other hand it is not strong enough to elute all proteins retained by *cis*-diol affinity interaction.

3.2 pH gradient

The pH dependency of PBC has been shown in the chapter III where interactions of different classes of proteins were obtained in the two conformations of PBA. Based on that, the pH gradient could be a useful strategy for proteins separation based on proteins

properties as presence of glycosylation or pI. Herein we studied two pH gradients based on Kröner et al. [31] studies: first at alkaline binding condition (pH 9 and 300 mM NaCl) for separation of glycoproteins (proteins retained by *cis*-diol interaction) and a second using acidic binding condition (between pHs 4 and 5) under low ionic strength for separation of acidic and neutral proteins retained by secondary interactions. The acidic binding condition was also performed using TAPS (tris varied) in order to enhance the selectivity and recovering. This compound is present in the original buffer composition of Kröner et al. [31] studies and although it avoids retention of glycosylated proteins at alkaline pHs TAPS does not decrease the proteins retention at acidic contions. On the other hand it can increase selectivity as soon as the pH increases. ProSep®-PB and Agarose P6XL were applied as chromatographic supports in order to compare their selectivity. All gradient provided a good linearity with high correlation coefficient (R^2): for the BGly buffer 0.9984 and 0.9951 for ProSep®-PB and Agarose P6XL, respectively. For BAN 0.9986 and 0.9995 (same for buffers 1 and 2) were obtained for the same supports, respectively with deviations lower than 0.0020 for all results. These values are comparable with the ones obtained by Kröner et al. [31] using MonoQ and MonoS columns (R^2>0.99).

3.2.1 pH gradient study for proteins retained by *cis*-diol interaction

The glycosylated proteins used in the pH gradient studies were also applied in this strategy plus RNase B as samples. According to the Figure 3 A, most of proteins were eluted at neutral condition when ProSep®-PB was used as matrix and cellulase came out only at cleaning step what was expected since this protein was adsorbed in this chromatography in all pH range from 4 to 9 in the adsorption studied of previous chapter. Comparing the supports, agarose matrix provided higher selectivity than ProSep®-PB eluting the proteins in different pH values as expected since agarose provided short pH range in the adsorption studies (Chapter III).

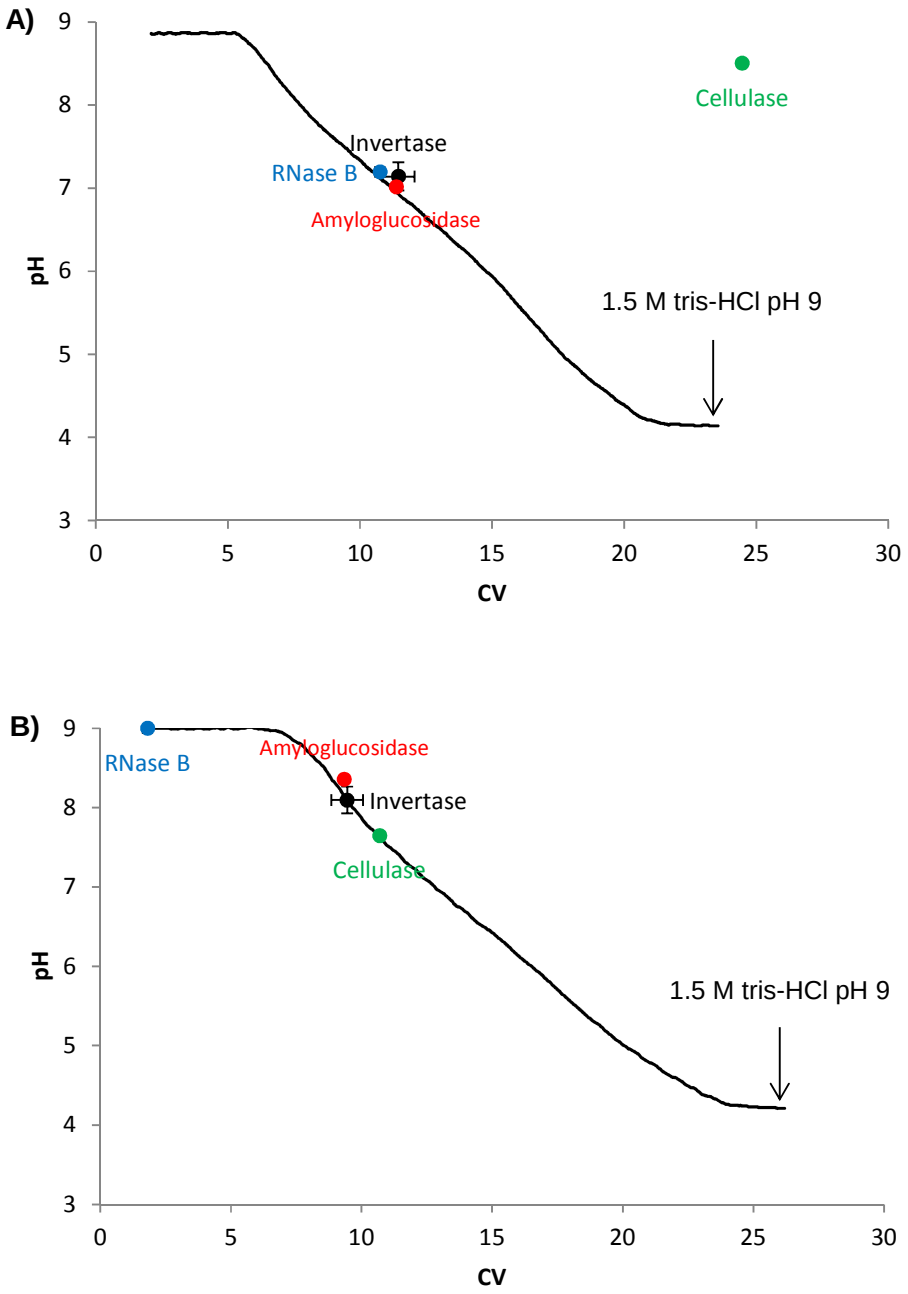

Fig. 3: pH gradient curves for capture of glycosylated proteins performed with BGly buffer based on Kröner et al. [31] work using ProSep®-PB (A) and Agarose P6XL (B) as matrices. 1 mg/mL of following glycosylated proteins were separately loaded in the column: invertase (black circle), amyloglucosidase (red circle), cellulase (green circle) and RNase B (blue circle). The elution point corresponds to the maximum value of the peak.

A problem related to pH gradient concerns to elute partially all proteins during the gradient, only invertase was completely recovered at gradient step when Agarose P6XL matrix was applied (Table 3). This behavior may be related to secondary interactions obtained when PBA changes the conformation from tetrahedral to trigonal. Interesting, RNase B and invertase did not bind under this conformation even though 9 % and 18 %, respectively, were still retained after gradient when ProSep®-PB was used. The effect of matrix effect could be responsible since this behavior was not observed with agarose matrix. Amyloglucosidase and cellulase had a similar behavior regarding the percentage of areas at agarose matrix, what was also expected since these proteins were adsorbed in almost all pH range of adsorption studies (chapter III) when NaCl was present. A drop of adsorption of amyloglucosidase around neutral pH (Figure 4 – chapter III) could be the reason for a small desorption in the pH gradient when ProSep®-PB was used.

Table 3: Percentage of peak areas of gradient and cleaning step from samples of pH gradient with BGly buffer for glycosylated proteins.

	Peak areas (%)			
Matrix	**ProSep®-PB**		**Agarose P6XL**	
Step	**Gradient**	**Cleaning**	**Gradient**	**Cleaning**
Invertase	67.9 ± 24.2	17.9 ± 25.2	80.3 ± 1.7	-
Amyloglucosidase	5.6 ± 0.2	93.2 ± 0.8	63.4 ± 14.8	26.3 ± 15.6
Cellulase	-	95.8 ± 0.4	46.4 ± 10.6	44.0 ± 11.0
RNase B	91.0 ± 2.2	9.0 ± 2.2	-	-

*****Percentage of flow through peak areas is included in the calculations.

3.2.2 pH gradient study for proteins retained by secondary interactions (Lewis acid-base, hydrophobic or aromatic)

An inverse pH gradient was also performed for proteins which had high adsorption at acidic binding condition. Amyloglucosidase, pepsin, cellulase, carbonic anhydrase and

conalbumin were the proteins selected from protein library for these studies. Different from previous pH gradient, *cis*-diol interaction can poorly occur under the binding condition used herein (between pHs 4 and 5). Lewis acid-base interaction is probably dominant but phenyl moiety probably plays a role performing hydrophobic and/or aromatic interactions depending on the surface properties of the protein.

Different from previous pH gradient studies (section 3.2.1), some proteins were split in two peaks during the gradient when BAN1 buffer was applied (Figure 4). Pepsin resulted in double peak in both matrices whereas carbonic anhydrase was split at ProSep®-PB matrix and conalbumin at Agarose P6XL matrix. As in the first pH gradient, cellulase was eluted only at cleaning step as well as conalbumin when ProSep®-PB was used. One possible reason for the doubled peaks is the impurities present since proteins are not 100% pure (Appendix A2). Although both matrices had split peaks, the elution of proteins were very different, all of them were earlier eluted in Agarose P6XL than in ProSep®-PB, both peaks. Besides, Agarose P6XL presented higher selectivity as expected. However, partial elution was also obtained by this pH gradient with most of proteins (Table 4) but agarose matrix provided higher percentage of recovery in the gradient than ProSep®-PB except when pepsin was loaded. These results could be related to the secondary effect of silica from ProSep®-PB which is not provided by agarose. Even when TAPS (tris derived) was used in the buffer composition (BAN2), partial elution was obtained for cellulase and carbonic anhydrase. This buffer component was responsible for not obtaining any retention of glycosylated proteins in the previous pH gradient (data not shown) due its tridentate interaction performed with PBA [25]. Under this condition, TAPS did not mitigate proteins interactions and an earlier elution and higher recovery during the gradient step was expected. Indeed, pepsin and cellulase eluted earlier than BAN1 buffer and higher recoveries were obtained during the gradient (Table 4).

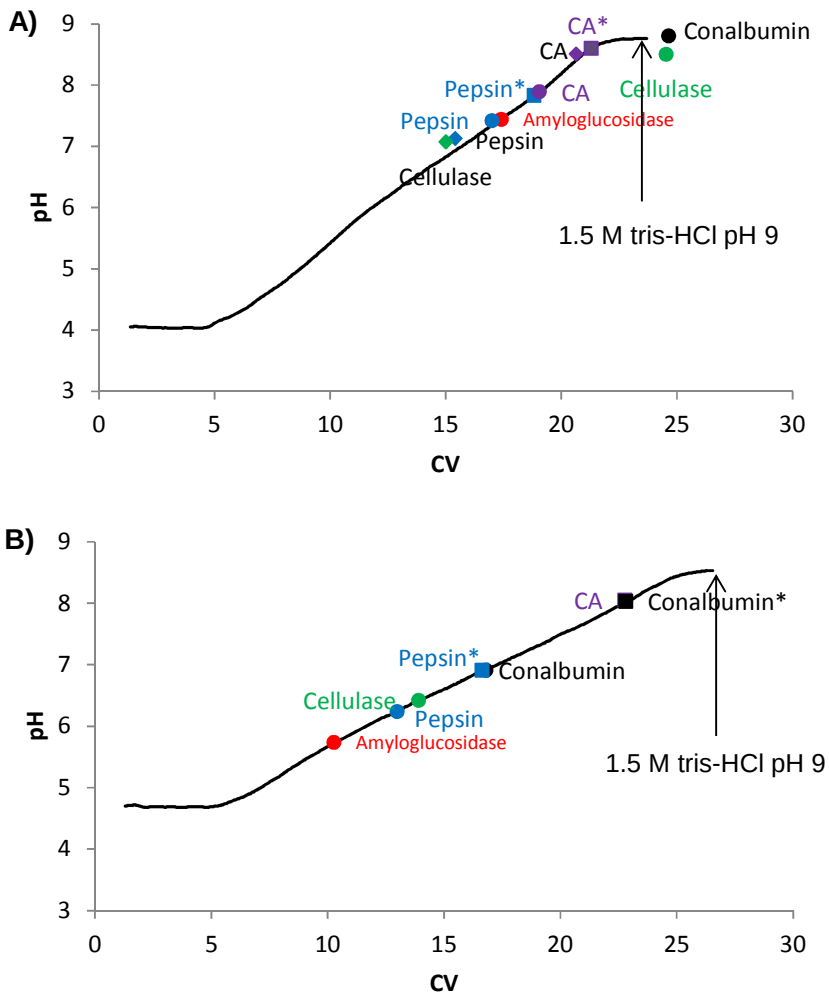

Fig. 4: pH gradient curves for capture of proteins interacted by secondary interactions named Lewis acid-base, hydrophobic and aromatic at pHs between 4 and 5. Process performed with BAN1 and 2 buffers based on Kröner et al. [31] work using ProSep®-PB (A) and Agarose P6XL (B) as matrices. 1 mg/mL of following glycosylated and nonglycosylated proteins were separately loaded in the column: amyloglucosidase (red), pepsin (blue), cellulase (green), carbonic anhydrase (purple) and conalbumin (black). Results from circles (first peak) and squares (second peak) are from BAN1 and diamonds from BAN2. Protein names with "*" correspond to the secondary peaks of these proteins. The elution point corresponds to the maximum value of the peak.

According to results obtained in the adsorption studies at chapter III, cellulase was adsorbed in all pH range studied in presence of NaCl but not at alkaline conditions without salt probably due to electrostatic repulsion. However, this repulsion is not strong enough in order to disrupt interaction between cellulase and PBA after binding, which was also already confirmed in the reverse gradient study. On the other hand, this protein was partially eluted when BAN2 was used. This probably occurred due to the presence of TAPS in the buffer composition.

Table 4: Percentage of peak areas of gradient and cleaning step from samples of pH gradient with BAN buffer for the acidic and neutral proteins: pepsin (PEP), amyloglucosidase (AMY), cellulase (CEL), carbonic anhydrase (CA) and conalbumin (CON).

	Peak areas (%)					
Matrix	**ProSep®-PB**		**ProSep®-PB**		**Agarose P6XL**	
Buffer	**BAN1**		**BAN2**		**BAN1**	
Step	**Gradient**	**Cleaning**	**Gradient**	**Cleaning**	**Gradient**	**Cleaning**
PEP	82.1±2.5/2.4±0.2[§]	5.0 ± 1.0	78.3 ± 15.7	-	60.7±8.2/4.1±0.9[§]	27.8 ± 8.1
AMY	37.7 ± 7.8	60.6 ± 8.1	NA[‡]	NA	77.5 ± 0.1	19.2 ± 0.8
CEL	-	98.5 ± 0.1	84.0 ± 3.4	7.8 ± 2.1	40.3 ± 5.4	57.6 ± 4.5
CA	10.3±2.5/1.0±0.2[§]	30.3 ± 15.1	14.7 ± 0.4	11.8 ± 6.1	23.5 ± 0.1	27.4 ± 1.1
CON	-	98.0 ± 1.3	NA	NA	28.5±1.1/18.1±0.1[§]	52.2 ± 3.3

*****Percentage of flow through peak areas is included in the calculations. [‡]Not analyzed. [§]Percentage of first and second peak, respectively.

3.3 Displacer gradient

A gradient of several modifiers were used to displace proteins retained by PBC: citrate, boric acid, tris-HCl, sorbitol, fructose, lactic acid and urea, all performed in a 20 CV of gradient from 0 to 500 mM of modifier, except urea with a gradient from 0 to 6 M. These processes were carried out for glycosylated (*cis*-diol interaction) at pH 9 with

supplementation of 300 mM NaCl in the binding buffer and for acidic and neutral proteins at pH 5 (mostly Lewis acid-base interactions). Only citrate and tris gradients were used for elution of processes with acidic binding condition.

3.3.1 Displacer gradient study for proteins retained by *cis*-diol interaction

All modifiers used have been described in the literature as able to disrupt protein retained on PBC [14,15,20,28]. Besides the ones discussed in the reverse gradient (section 3.1), sorbitol and fructose are *cis*-diol saccharides able to esterify with PBA. These both saccharides provide high association constants for this esterification (1000 M^{-1} and 560 M^{-1} at pH 8.5, respectively) [13]. Lactic acid, as citrate, is also an α-hydroxyacid able to perform charge transfer by Lewis acid-base interaction or chelate by cyclic interaction with PBA [34]. According to these authors, this interaction can occur in a large pH range from 2 to 12. The acidic (invertase and amyloglucosidase), neutral (cellulase) and basic (RNase B) glycosylated proteins were used as samples by separated loadings.

Among the modifiers applied, citrate, boric acid and urea were not able to elute any glycosylated proteins used in these studies. All of them were fully recovered at cleaning step with 1.5 M tris. The inefficiency of citrate must be related to its low interaction with PBA at tetrahedral conformation by charge transfer since this acid has to be harder on electronegativity than hydroniums of aqueous phase to interact [10]. According to Smith and March [29], both carboxylic acid and hydroniums are hard Lewis bases but the latter is more electronegative than the acid. However, lactic acid was able to elute the proteins proving to be a harder Lewis bases than citrate, but a problem associated with the application of this acid was obtained. Lactic acid somehow over packed the column blocking the porous of beads which became inappropriate for further utilization, one hypothesis is a possible polymerization of this acid under alkaline pHs since lactic acid can also be used as a monomer in lactide form (cyclic di-ester of lactic acid) [35]. Although

some selectivity could be reached by lactic acid, it may not be appropriate for using at ProSep®-PB under this condition. On the other hand, tris, sorbitol and fructose eluted all proteins recovering 100%. According to the results presented at Figure 5, sorbitol and fructose did not provide any selectivity since all proteins eluted at the same concentration during the gradient. On the other hand tris eluted selectively the proteins applied. As a shielding reagent, tris can disrupt not only *cis*-diol interactions but also secondary interactions which may play a role in the retention of these proteins. Although sorbitol has been reported as able to elute nonspecific interaction as well [15], it was not selective in this study.

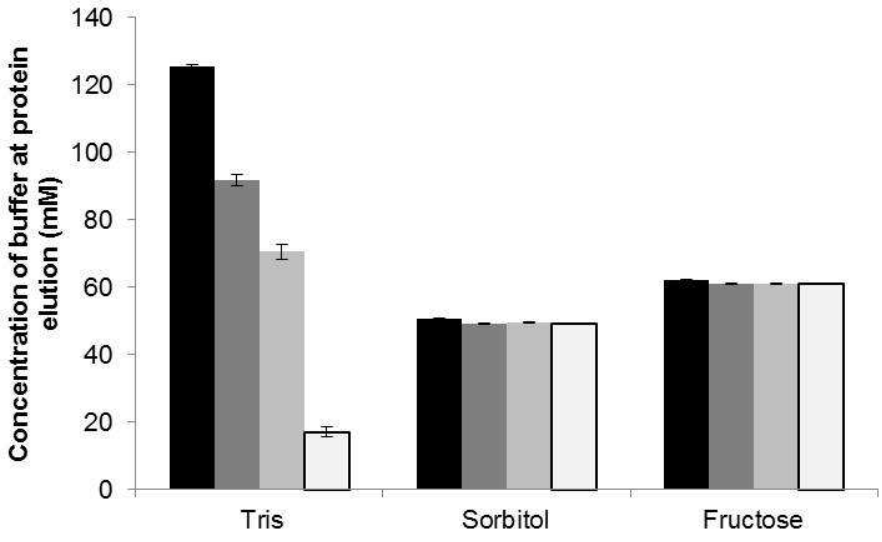

Fig. 5: Concentration of elution buffer at maximum value of protein elution peak area of acidics glycosylated proteins invertase (■), amyloglucosidase (■), neutral cellulase (■) and basic RNase B (□) using tris, sorbitol and fructose as displacers in a gradient ranging from 0 to 500 mM of concentration at pH 9 with 20 mM CHES supplemented with 300 mM NaCl as binding condition. The displacers were prepared in the binding buffer. These values correspond to complete elution.

Based on these results, 100 µL of an artificial mixture of 0.25 mg/mL from the glycosylated proteins of amyloglucosidase, cellulase, RNase B and 17.5 µg/mL invertase were prepared for loading in the column in order to obtain the separation profile. The same gradient, 20 CV to 100% of elution buffer, was initially performed. Three peaks at elution were obtained but not completely separated (Figure 6 A – chromatogram 1). In order to enhance the selectivity different gradient slopes were carried out using the same buffer 0.5 M tris. The processes were performed by lowering the gradient rate until the peaks obtained were completely separated. It is already known by the literature the enhancement of chromatography efficiency when lower gradient rates are applied [21,36,37], salt gradient slopes are usually performed for ion exchange chromatography although the use of modifiers can also improve the selectivity [36]. From 4.72% of tris/min (first process) 4 more experiments were carried out and the best selectivity was obtained at gradient rate of 0.57% tris/min. After confirming the fifth gradient slope with a replicate, a process with 2.5-fold higher loading was carried out and the same profile was obtained. Fractions from this process were analyzed by SDS-PAGE (Figure 6 C) and comparing with electrophoresis gel of pure commercial samples (Figure 6 B). It can be observed that proteins were eluted in the same sequence as when separated loadings were performed. RNase B was almost completely eluted in the first peak followed by cellulase in the second peak with low contamination of RNase B and amyloglucosidase at peak 4. Although invertase was also added, it did not appear in the gel. This protein was probably too diluted in the long tail after fourth peak and fractions obtained at this point did not reveal the presence of any protein (data not shown). Although the peaks were separated, they were highly diluted as can be observed in the Figure 6. One solution for increasing the peak resolution and consequently decreasing the dilution of separated proteins could be a performance of stepwise elution based on tris concentration obtained in the maximum values of the peaks [23].

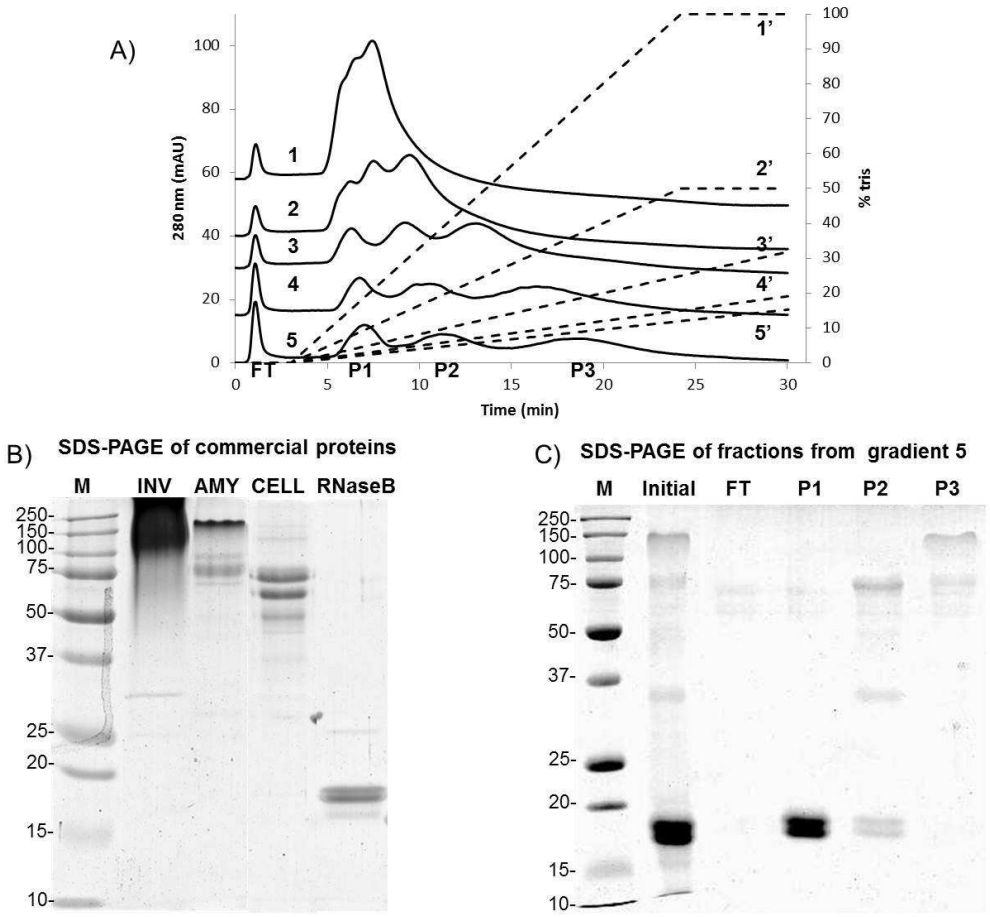

Fig. 6: Different slopes of 0.5 M tris gradient using an artificial mixture of 0.25 mg/mL of following glycosylated proteins amyloglucosidase, neutral cellulase and basic RNase B and 17.5 µg/mL of invertase. A) Chromatograms of different tris gradient rates at pH 9 with 20 mM CHES supplemented with 300 mM NaCl as binding condition. Chromatogram 1 were obtained from the 4.72 % tris/min (1'), 2 from 2.36 % tris/min (2'), 3 from 1.18 % tris/min (3'), 4 from 0.71 % tris/min (4') and 5 from 0.57 % tris/min (5'), 4 peaks were obtained from the fifth process, one from flowthrough (FT) and 3 from elution (P1 to P3). B) SDS-PAGE of commercial protein samples separated loaded, invertase (INV), amyloglucosidase (AMY), cellulase (CELL) and RNase B. C) SDS-PAGE of peak fractions FT, P1, P2 and P3 plus initial sample loaded in the column and marker (M) from the 5th gradient performed with 250 µL of volume loaded in the column.

3.3.2 Displacer gradient study for acidic and neutral proteins retained by secondary interactions (Lewis acid-base, hydrophobic/aromatic) at lower pH

A similar study was carried out with acidic and neutral proteins using 50 mM acetate at pH 5 as binding condition. The same process was performed with citrate or tris as modifiers. Pepsin, amyloglucosidase, cellulase, CA and conalbumin were selected for these studies since they provided adsorption under this condition in the previous chapter III. According to the results, *cis*-diol is not the dominant interaction under this condition but Lewis acid-base seems to play the main role based on mitigation of proteins adsorption by citrate in different concentrations, a hard Lewis base. Indeed, when this acid was used in the gradient most of proteins were eluted but not CA and conalbumin (Figure 7). Although CA required higher concentration (200 mM) than pepsin (10 mM) or cellulase (50 mM) of this acid for obtaining a decrease on adsorption (Figure 5C – chapter III), the elution up to 0.5 M citrate was not high enough to desorb CA and conalbumin. The sequence of elution matches with results from adsorption studies, since pepsin had higher effect on adsorption compared to cellulase and CA.

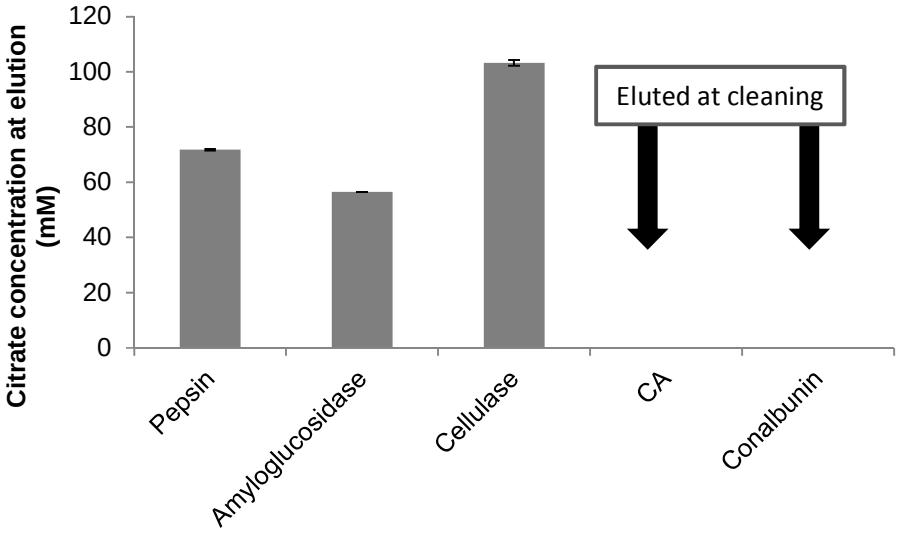

Fig. 7: Concentration of elution buffer at maximum value of protein elution peak area of pepsin, amyloglucosidase, cellulase, carbonic anhydrase (CA) and conalbumin using citrate as displacer in a gradient ranging from 0 to 500 mM of concentration at pH 5 with 50 mM acetate buffer as binding condition. The displacers were prepared in the binding buffer. These values correspond to complete elution.

Citrate is an α-hydroxyacid with three carboxylic acids and a *cis*-diol group in its structure. Besides the ability of performing Lewis acid-base interaction, the negatively charged carboxylates of its structure (Figure 6 – Chapter III) could provide electrostatic interactions or repulsion of negatively charged proteins after complexation with PBA. This fact could be responsible for the selectivity obtained with citrate. A study with RNase A (basic nonglycosylated protein) was performed in order to confirm this hypothesis. This basic protein does not interact with PBA at pH 4 when acetate buffer is used (Chapter III). Based on that, adsorption studies with RNase A under different binding conditions using citrate from 10 to 200 mM of concentration at pH 4 was carried out. According to results of Figure 8, RNase A was retained at pH 4 in all concentrations of citrate used in a similar binding capacity as positive control (pH 9). 300 mM NaCl was not able to shield this

interaction (grey bars of Figure 8). Only a small effect can be observed when 10 mM citrate was used, although the salt does not mitigate completely the interaction. This effect can explain why CA and conalbumin did not elute with 500 mM citrate since they are neutral proteins (pI 6.6 and 6.1, respectively) and positively charged at pH 5. However, cellulase with pI 7.7 is also positively charged and it was eluted by the same process. According to adsorption studies at chapter III, lower concentration of citrate was necessary to mitigate cellulase adsorption compared to CA. Also studies of CA protein surface properties reveled that hydrophobic patches near negative amino acids could probably be responsible for strengthening the interaction, what was not observed for cellulase, a highly hydrophilic protein.

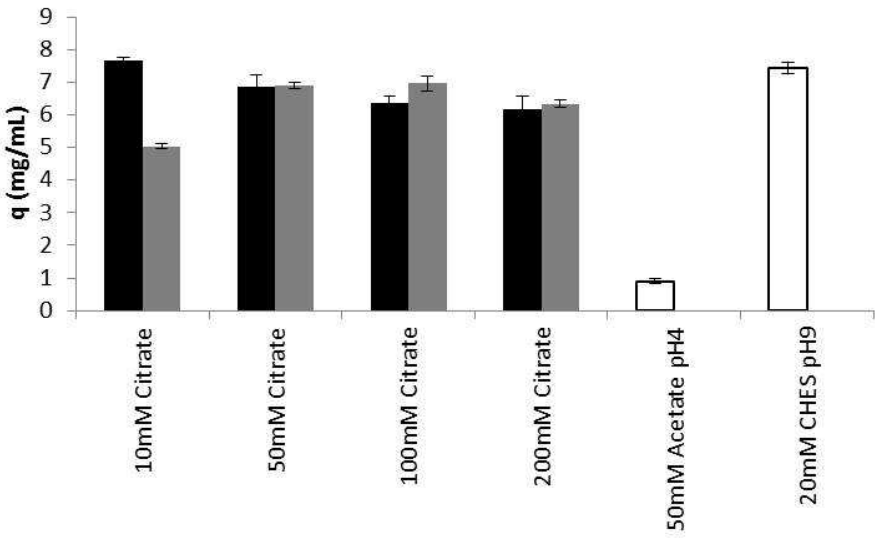

Fig. 8: Binding capacity (q) at equilibrium of RNase A in Prosep®-PB matrix in different concentrations of citrate at pH 4 in the presence of 0 mM (■) and 300 mM (■) NaCl. 50 mM acetate pH 4 was used as negative control and 20 mM CHES pH 9 as positive control.

Exploring the citrate selectivity, an artificial mixture of 0.25 mg/mL of the proteins, except conalbumin, was also loaded in order to set the best gradient rate of citrate for proteins separation. As in the previous gradient study, 100 µL of this mixture was loaded

and with a gradient rate of 4.76% citrate/min a selective separation of the proteins during elution was not achieved. As tris gradient studied, here the best selectivity was achieved with 0.57% citrate/min of gradient rate (Figure 9). A higher loading with 250 µL of the same sample was performed and the process provided a similar profile.

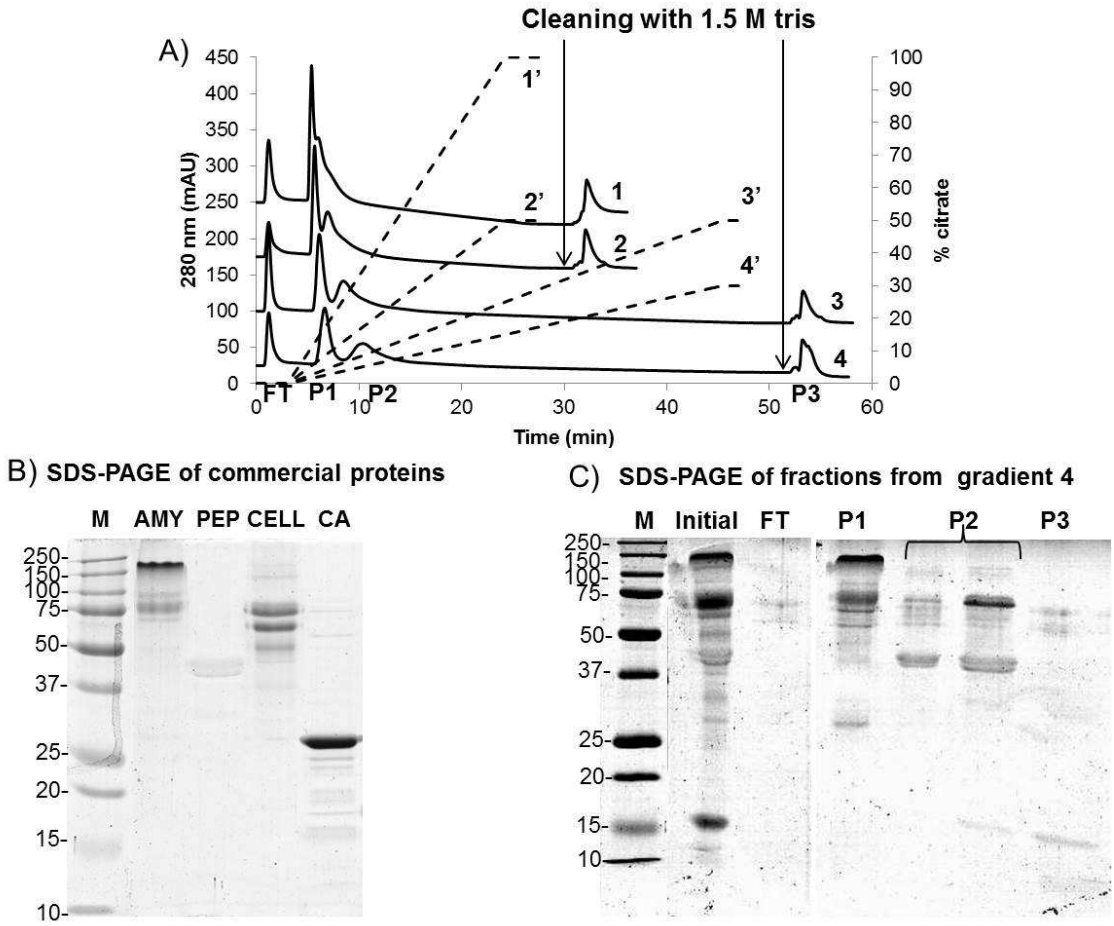

Fig. 9: Different slopes of 0.5 M citrate gradient using an artificial mixture of 0.25 mg/mL of following proteins amyloglucosidase, pepsin, cellulase and CA. A) Chromatograms of different citrate gradient rates at pH 5 with 50 mM acetate buffer as binding condition. Chromatogram 1 were obtained from the 4.72% citrate/min (1'), 2 from 2.36% citrate/min (2'), 3 from 1.18% citrate/min (3'), 4 from 0.57% citrate/min (4'), 4 peaks were obtained from the fourth process (FT and P1 to P3). B) SDS-PAGE of commercial protein samples separated loaded, amyloglucosidase (AMY), pepsin (PEP), cellulase (CELL) and CA. C) SDS-PAGE of peak fractions flowthrough (FT), P1, P2 (two fractions, first from maximum peak value and second from the tail formation in the end of the peak and P3 plus initial sample loaded in the column and marker (M) from the 4th gradient performed with 250 µL of volume loaded in the column.

According to the gel electrophoresis of peak fraction from the gradient slope 4 (Figure 9 C), the initial protein sample shows more than 4 bands which are probably impurities present in these commercial proteins, although the majority are the target proteins (darker bands), except for a band around 15 KDa. It can be observed that at flow through step (P1) cellulase was the protein mostly present. The first peak of elution (P2) is mostly composed by amyloglucosidase, however CA seems to be eluted in this fraction by the presence of a band with same molecular weight as the band of CA commercial (Figure 9 B). The presence of CA was not expected here since this protein was not eluted by citrate gradient when it was separately loaded. Protein-protein interaction could be probably the reason for this behavior since amyloglucosidase is mostly negatively charged and CA positively charged under this condition. The third peak was presented in two fractions, one obtained in the maximum peak value and second from the tail in the end of the peak. Pepsin is present in both fractions as expected, however a band with higher concentration of a higher molecular weight (75 kDa) is obtained in the second peak, probably cellulase. Cellulase has two major bands when pure load was performed at SDS-PAGE, and both seems to be present in all peak fractions, although in the second fraction of P3 seems to have at higher amount compared to the other bands. A band presents at considerable amount around 15 KDa in the initial sample does not match with any band from commercial proteins used. This protein was partially eluted in P3 (second fraction) but it is mostly present in the P4, eluted along with cellulase.

Tris was the second modifier used in the gradient process for protein capturing at lower pH. In the first process carried out, 0.5 M tris was prepared at pH 5 with 50 mM acetate. Under this condition the amine moiety of tris is protonated and probably interacts with deprotonated carboxylic acid of acetate. This salt formation somehow affects the trident interaction with PBA under this condition since the proteins, same as used with citrate concentration, were not eluted by tris gradient under this condition. In the

adsorption studied, tris was used at pH 8.5 when amine is deprotonated and the disruption occurred for all proteins. Based on that, this condition was adopted here, using 0.5 M tris pH 8.5 in a 20 CV gradient. The elution was successfully obtained. The proteins were 100% recovered although this gradient did not provide any selectivity, all proteins were recovered in a short concentration range from 86 to 95 mM of tris. The range of concentration of elution is related to the abrupt increasing of pH from 5 to 8.5 as well as the conductivity due the salt formation until tris reaches its pKa (around pH 8).

4. Conclusions

This work has clarified some insights focusing on multimodal and pH-dependent behavior of phenylboronate chromatography. Generally we can conclude that this chromatography can specifically retain certain classes of proteins in both configurations, glycosylated proteins at tetrahedral configuration usually performed at alkaline conditions and proteins with considerable negatively charge (mainly provided by carboxylic acids) in conditions where PBA is at trigonal conformation, usually acidic and neutral proteins. Based on these conclusions we tried to prove this concept using three strategies of elution in order to enhance the selectivity of this chromatography: reverse gradient, pH gradient and displacer gradient.

Separation of acidic from neutral glycosylated proteins by reverse salt gradient strategy was not entirely achieved, although the presence of 15 mM tris or the presence of HEPES buffer, increased selectivity. Also, it was not possible to obtain 100% proteins recovery probably due to electronegative charges repulsion, although, charge repulsion avoids proteins binding. Thus, *cis*-diol esterification is strong enough to hold the proteins upon this condition. Secondary interactions named hydrophobic and hydrogen bond could

also play a role in interaction strength, even though, the results indicated the mechanisms of adsorption are probably distinct from desorption.

The use of pH gradient was not a properly separation tool as expected. The multimodal interaction of PBA is the main reason for not obtaining 100% of proteins recovering during the gradient. The proteins were mostly eluted at neutral pH, between pH 7 and 8 when the conformation changing of PBA can happen. This range of elution is more common when ProSep®-PB matrix is applied since Agarose P6XL provided a larger range of protein elution, even though agarose did not provide 100% of protein recovering in the gradient elution. These results can also indicate that the pH dropping is not the best elution option for PBC chromatography. This fact has already been reported in the literature for IgG purification with only 7% recovering when pH drop to 2 was performed [14]. Although recoveries about 90% were obtained when this strategy was applied for elution of nucleosides by extraction with PBA immobilized in gel [38].

Displacer gradient was the most selective method applied not only for glycosylated proteins at usual alkaline conditions but also for proteins binding at lower pH range where Lewis acid-base is mostly the dominant interaction. Testing the usual displacers for PBC, sorbitol and fructose, efficient recovering of 100% were obtained but were not selective for all the different glycosylated proteins. On the other hand, tris provided not only total recovery but also the best selectivity for glycosylated proteins. For proteins adsorbed at acidic condition, tris also eluted with full recovering when applied at pH 8.5. However this condition has found to not be appropriated for obtaining selectivity due to the difficulty on controlling process conditions as pH and conductivity. Under this binding condition, citrate provided a good selectivity as well as efficiency on recovering 100% some of the proteins. This ability is probably related to the interaction with PBA by Lewis acid-base interaction and chelating interaction by α-hydroxylate moiety, as well as providing electrostatic repulsion by negative charge of carboxylates present in its structure.

This work has proved not only the concept of multimodal interaction but also the wide options of implement selectivity of this chromatography by playing with different modifiers. At lower pH more carboxylates could be used depending on the application used as well as tris derived at alkaline conditions could improve the selectivity depending on the separation. Insight on the mechanisms of interaction has also been demonstrated. These unique features make PBC a useful tool not only for separation but also for analytical purposes and can be highly explored for deeper separations as proteins isoforms or proteomic studies.

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CHAPTER V

PHENYLBORONATE CHROMATOGRAPHY FOR ANTIBODIES SEPARATION: ADSORPTION STUDIES AND CRYOGEL FUNCTIONALIZATION FOR PROCESS INTEGRATION

Results presented in the following conference:

Carvalho, R.J., Azevedo, A.M., Cramer, S.M., Aires-Barros, M.R. Purification of antibodies using continuous bed chromatography with cryogels monolith support – Affinity, Tavira/Portugal, 2011.

Phenylboronate chromatography for antibodies separation: adsorption studies and cryogel functionalization for process integration

Abstract

In this work commercial phenylboronate matrices based on methacrylate or agarose were used to specifically bind a polyclonal antibody regarding a mixture of contaminants proteins and to determine the best binding conditions in order to obtain high recovery and selectivity. Furthermore, immobilizing phenylboronic acid onto cryogels monolithic supports with functional epoxy group were tested in order to further purify monoclonal antibodies by integrating clarification and capture steps. HEPES buffer at pH 8.5 was selected as the best binding conditions for agarose matrix. The best results in terms of performance and selectivity were also obtained with this matrix. Different functionalized cryogels with several derivatives of phenylboronic acid were analyzed by adsorption of polyclonal IgG at 20 mM HEPES pH 8.5 and by colorimetric analysis using alizarin red S dye in order to obtain the ligand density. The immobilization of 3carboxyl and formyl derivatives performed at pH 8 presented better efficiency on IgG binding, but no relation was found between IgG adsorption and ligand density. Among all reactions, mercapto and 3carboxyl derivate provided the best IgG adsorption (around 1.5 mg of IgG/mL of cryogel) when hexamethylenediamine and ammonia were used as space-arms, respectively.

*Keywords***:** phenylboronate chromatography, monoclonal antibodies separation, cryogels, monolith supports, phenylboronic acid, immobilization

1. Introduction

The use of antibodies for therapeutic purposes has grown exponentially in the last decade and in the last year has become a market of 60 billion dollars with an expectation growth of 15% until 2018 [1]. The potential of antibodies as therapeutic agents explains the fast market growth in only two decades and the interests of public and private investors in mAbs research which resulted in the development or several drugs against immune diseases, transplant rejections and several types of cancer [2]. We can currently find 34 approved drugs from Food and Drug Administration American agency and several others in clinical trials [2]. According to the literature, high doses of mAbs as therapeutic agents are necessary in order to obtain an immune response to fight several diseases, especially chronic ones [3]. Many efforts have been made in order to improve mAbs production processes and nowadays high titers reaching more than 25 g/L by production using Chinese hamster ovary (CHO) cells has been referred [4]. Although CHO cells are the most popular for mAbs production, studies with other mammalian cells (NS0, Sp2/0, PER.C6) have been also referred in the literature [5].

Due to cell culture developments over the last 10 years the process bottleneck is now on the purification side. Therefore, there is a need of new technologies and more efficient, robust and high capacity processes. Several studies on antibodies purifications are described in the literature using different types of chromatography such as multimodal [6], anion exchange [7], hydrophobic [8] and affinity with protein A [9]. The established downstream process includes 3 main steps, protein A as capturing step and two remain steps for viruses removal [10]. Protein A from *Staphylococcus aureus* cell wall interacts mostly with the Fc region of the antibody and a high selectivity and purities higher than 95% are obtained in one step [11]. Protein A chromatography is easy to implement, robust and flexible regarding conductivity and pH facilitating the purification process as no special

sample preparation is needed as in anion exchange and hydrophobic chromatography processes [9,11], however an industrial level protein A chromatography accounts for more than 50% of total costs of the entire mAbs downstream process [3,12]. Besides, protein A ligand can be very unstable limiting its applicability and thus more stable and cost effective ligands are needed. Synthetic ligands have been used namely peptides [13] and *de novo* designing artificial ligands trying to mimetic protein A affinity [14]. Moreover, multimodal ligands are currently being widely applied on mAbs downstream process due to its ability to interact through more than one via conducting to higher selectivity and capacities [6]. Hydroxyapatite [15], hydrophobic charge induction (HCIC) [16] and phenylboronate chromatography (PBC) [3,17] are examples of multimodal chromatography applied on mAbs separation and purification. This type of chromatography can thus create a unique selectivity under certain conditions. For instance, phenylboronic acid (PBA) can be found in two different conformations, trigonal and tetrahedral at alkaline conditions due to its ability to coordinate Lewis bases as free hydroxyls [18]. According to the literature, higher associate constants of esterification with *cis*-diol compounds present in saccharides, RNA, nucleosides, α-hydroxydes and glycoproteins as mAbs [19] are obtained at tetrahedral configuration. PBA is thus a potential candidate to replace Protein A due to the higher chemical stability and lower cost, specifically binding and the multimodal behavior.

Several alternatives strategies and methodologies to the traditional process for mAbs purification have been proposed and developed , among them we can highlight the aqueous two phase systems [20] expanded bed chromatography [10] membrane chromatography [21], magnetic particles [12] and monolith chromatography [22]. Compare to packed bed chromatography these alternative strategies allow process integration thus reducing the costs of mAbs downstream process. Monoliths, called the fourth chromatography generation, are single blocks bed chromatography with large porosity which allows separation of large molecules as proteins, proteins aggregates, DNA, viruses

and even cells [23]. Monoliths can operate at higher flow rates than packed bed without changes in the dynamic binding capacity due to the convective transport along the bed [23]. Cryogel is an example of a monolith support with large porous up to 100 μm of size. The name cryogel is due to the way it is produced: the polymerization occurs at very low temperatures, around -12ºC, and the frozen water crystals formed are the responsible for the porous channel formation [24]. The easy and low cost production of cryogel has caught the attention of several research groups for its application on the separation of large molecules like cells [25]. The flexibility of cryogel when hydrated (sponge aspect), which allows its compression during elution, can be used to improve the efficiency of desorption of large molecules as cells [24]. This support has been widely applied on PBC in the last years [26]. Attempts to obtain different cryogels functionalized with PBA have been carried out via direct reactions by copolymerization using vinylphenylboronic acid (VPBA) or aminephenylboronic acid (APBA) or via indirect reactions by several PBA derivatives using cryogel with several functional groups (epoxide, carboxyl or aldehyde) [26]. Studies with copolymerized cryogels have demonstrated higher ligand density in the case of direct reactions, although, the ligands are not freely available for large molecules as cells which difficult their recognition but smaller molecules as RNA are able to recognize and bind [27]. Cryogel supports functionalized with PBC have been also applied in capillary chromatography with better efficiencies [28].

In this work, adsorption studies of polyclonal antibodies have been performed. Human serum albumin and recombinant human insulin were also applied in these studies since they have been reported as the main impurities of cell culture supernatant of antibodies production [3]. The adsorption studies were carried out using phenylboronate commercial matrices based on polymethacrylate (boric acid gel) and agarose (Agarose P6XL), under different binding conditions, in order to increase process selectivity. In the second part of the work, immobilization of different PBA derivatives onto cryogel with

functional epoxy groups using space-arms and cross-linkers were carried out in order to obtain high ligand density for IgG and simultaneously to allow integration of clarification and capture steps of mAbs downstream process.

2. Materials and Methods

2.1 Materials and chemicals

Human polyclonal IgG for therapeutic purposes named Gammanorm was purchased from Octapharma (Lunch, Switzerland). 3-Formylphenylboronic Acid (FPBA); 3- and 4-Carboxyphenylboronic Acid (3- and 4-CPBA), 3-Aminophenylboronic Acid (APBA), Mercaptophenylboronic Acid (MPBA), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (CD), ethylene glycol (EG), glutaraldehyde 50% solution grade I (GD), 1,4 butanediol diglycidyl ether (BDGE), aniline, glycine, 8-Anilino-1-naphthalenesulfonic acid (ANS), sodium phosphate mono and dibasic anhydrous, sodium citrate anhydrous, sodium acetate anhydrous, sodium fluoride, tris(hydroxymethyl)aminomethane (Tris), sodium chloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2-(4-morpholino)ethanesulfonic acid (MES), hexamethylenediamine, ethylenediamine, boric acid, borax anhydrous, phosphate saline buffer (PBS) and human serum albumin (HSA) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Recombinant human insulin named ActRapid was obtained from Novo Nordisk (Copenhagen, Denmark) and 4-mercaptoethyl pyridine (MEP) was purchased from Carbosynth (Berkishire, UK).

The chromatographic medias used in this study were Agarose P6XL (Appendix B2) from ProMetic Biosciences (British Isles, UK), Boric acid gel methacrylate based with aminophenylboronic acid functionalized (Appendix B3) from Sigma-Aldrich® (St. Louis, MO, USA) and Cryogel BasicE (0.5 and 1. 6 mL sizes), a macroporous monolith methacrylate based with functional epoxy groups (Appendix B4) from Protista (Lund,

Sweden). All reagents used had a purity ≥ 95% and they were PA or of HPLC grade. All the water used in the experiments was obtained from MilliQ purification system (EMD Millipore, USA).

Äkta Purifier systems (GE Healthcare, Uppsala, Sweden) were used to perform all column experiments, both systems were operated online by the software Unicorn 5.11. Tricorn™ 5/20 (i.d. 5 mm; max. bed height 29 mm) empty glass columns were used for packing Boric acid gel and Agarose P6XL to a column volume (CV) of 400 µL and 10/20 (i.d. 10 mm; max. bed height 29 mm) columns for packing the cryogels with 1.6 mL of CV.

2.2 Adsorption Experiments

2.2.1 Column Experiments

Proteins were separately loaded in amounts of 500 µg for IgG and insulin and 1 mg for HSA into Boric acid gel and Agarose P6XL packed columns. Different binding conditions were performed using different buffers in order to analyze their effects: PBS (10 mM phosphate + 150 mM NaCl), 20 mM HEPES + 150 mM NaCl, 50 mM borate + 150 mM NaCl were the buffers used in the binding condition in the following pHs at Table 1. These experiments were performed for both columns.

Table 1: Binding conditions for adsorption experiments in columns packed with boric acid or Agarose P6XL.

	PBS	HEPES	Borate
pH 7.5	✓	✓	✓
pH 7.9	✓	✓	✓
pH 8.5		✓	✓
pH 9.0			✓

Elution of adsorbed proteins was carried out by changing the eluent to 1.5 M tris-HCl pH 8.5 under a 10 CV gradient elution based on previous work performed using ProSep®-PB, a controlled-pore glass beads functionalized with APBA [3]. According to these authors, this eluent was able to recover all proteins bound.

The percentage of protein adsorbed was determined based on area of eluted peak divided per total peak area in the chromatogram. The experiments were carried out in duplicate.

2.2.2 Dynamic binding capacity (DBC) by frontal analysis

The frontal analysis was performed using 4 mg/mL of polyclonal IgG diluted in the binding buffer. The supports used were 0.4 mL of Agarose P6XL or Boric Acid gel packed in glass columns. The binding condition was carried out at pH 8.5 using 20 mM HEPES buffer supplemented with 150 mM NaCl or 300 mM NaCl. The flow rate of 1 mL/min was used for loading the sample and elution studies. The experiment was carried out at Äkta system connected with a super loop of 50 mL. The void volume (V_0) was determined by loading 50 mM phenol red. The DBC (q_{10}) was calculated at 10% of breakthrough curve by the following equation 1:

$$q_{10} = \frac{C_0(V_{10} - V_0)}{V_B} \quad (1)$$

2.3 Immobilization of PBA on cryogels

Several reactions were performed for immobilization of PBA on cryogels as shown in the Figure 1. Cryogel BasicE contains 30 µmol/mL of functional epoxy groups, according to the supplier, and activation was performed with ammonia or amine space-arms (ED or HMD) at alkaline pH. After activation, the crosslinkers were reacted in order to link the amine to the target ligand. Some direct reactions were also performed without a cross-

linker depending on the PBA functional group. The reagent concentrations were calculated based on cryogel functional epoxy groups. The space-arms and cross-linkers reagents were reacted under 10-fold excess compared to epoxy groups and ligands 50-fold, except 3- and 4-CPBA (10-fold) due its low solubility in aqueous medium. All reactions performed were carried out under constant circulation of 1 mL/min by ISMATEC tubing pump MS-CA serie with 4 tube channels from IDEX Health & Science (Wertheim, Germany). The details of these reactions are described below.

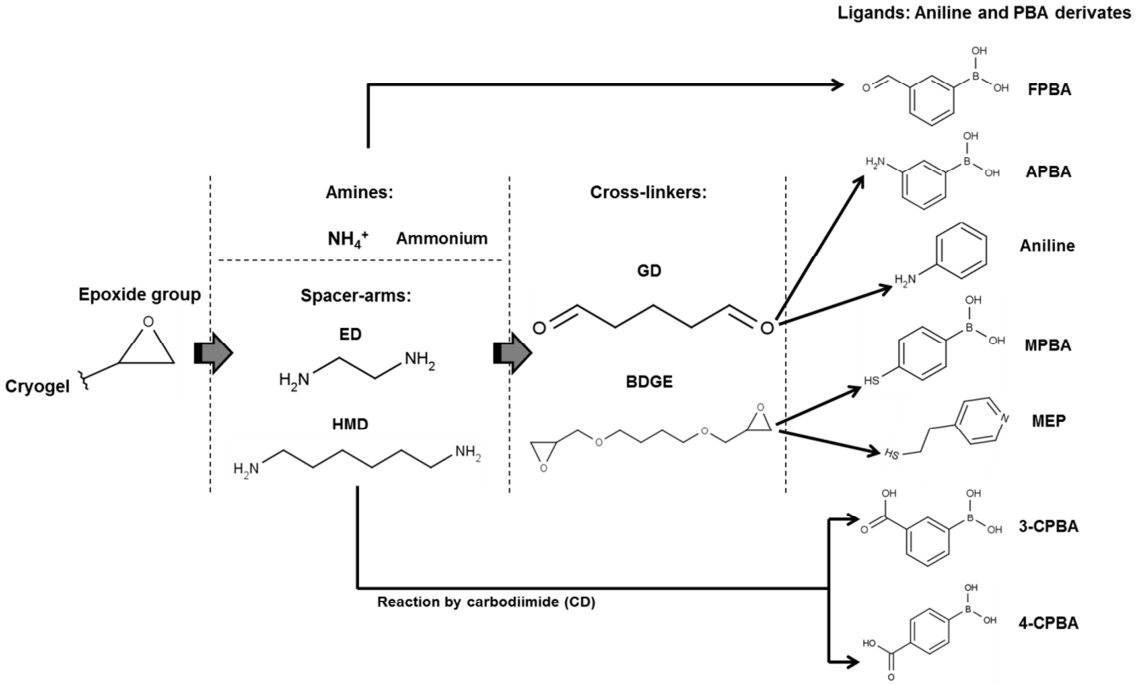

Fig. 1: Scheme of all reactions performed: amination by ammonium and space-arms, cross-linkers reactions and ligands immobilization. The ligands used for immobilizing onto cryogels supports were PBA derivates and also with aniline or MEP. The reactions steps were performed according to ligands functional group.

The amination reaction of epoxides by ammonium or spacer-arms (ED and HMD) was carried out for 24 h at room temperature (RT). The reactions were conducted in alkaline pH, namely at pH 10.5 for 14% ammonium and at pH 9.5 for the two spacer-arms

under constant circulation according to Cryogel BasicE supplier information. Samples from these reactions were maintained without further reactions in order to analyze the effect of amine and space-arms on IgG adsorption.

After amination, 3-formylphenylboronic acid (FPBA) was directly reacted with amino groups from space-arms and ammonium at two different pHs, 4.5 and 8.0 for 4 h at room temperature (RT) [29]. All reactions were performed in aqueous medium.

3- and 4-carboxyphenylboronic acid (3- and 4-CPBA) were used in a direct reaction using carbodiimide (CD) in order to reacting carboxyl with amino group from spacer-arm or ammonium. The reactions were performed at the same pHs as FPBA and in two steps: 3- or 4-CPBA reaction with carbodiimide followed by the reaction of the product obtained with the amine [29]. CD and CPBA were reacted for at least 4 h at RT and the cryogel was added to the reaction and maintained for more 15 h at RT.

The immobilizations with aniline, MPBA and APBA were performed in 3 steps of reaction, first amination with ammonia and space-arms, then the cross-linkers were reacted, 1,4 butanodiol diglycidil ether (BDGE) for immobilization of MPBA and glutaraldehyde (GD) for immobilization of APBA and aniline.

The BDGE cross-linker was reacted for 24 h with cryogel aminated and MPBA at alkaline pH 9.5 for 24 h under circulation and controlled temperature of 28ºC. These conditions were all based in the literature of thiol reactions [30].

APBA, the most common PBA derivate used in functionalization of supports for boronate chromatography, was also used in these studies. As discussed before, GD was the cross-linker used after activation of epoxy group with space-arms and ammonia. Each reaction for GD and later for APBA was performed according to Cryogel BasicE supplier information at pH 7.2 for 24 h at RT. Other two different reactions for APBA immobilization were carried out. In the second, glycine was first reacted with epoxy groups at pH 8.0 for 48 h at RT. After that, CD solution was added into cryogel at two different pHs 4.5 and 8.0

and reacted for 24 h at RT. APBA was later added and let to reacted for 15 h at least. The third reaction was performed according to Dukler and Freeman conditions [31]. APBA was dissolved in a solution of 30% ethylene glycol pH 8.0 and this solution was reacted with the cryogel for 96 h at RT. The same reactions and conditions were used with aniline in order to understand the influence of aniline moiety of PB in the adsorption with and without a spacer-arm.

2.4 Functionalized cryogels assays

Two assays were used in order analyze the immobilization efficiency of PBA derivatives: alizarin red S (ARS), an organic red dye reported as a *cis*-diol compound with high affinity with PBA [19] and binding of polyclonal human IgG. For cryogels only aminated by ammonium or space-arms or aniline and MEP functionalized only the IgG binding assay was performed. The details of these assays are described below.

2.4.1 APBA ligand density measurement by ARS

The ligand density was determined using Alizarin Red S (ARS) adsorption to phenylboronic acid [32]. The assay was performed at pH 7 using all cryogels functionalized with PBA in tubes with 6 CV of dye solutions. The same volumes of washing and elution buffer were used. The concentration of dye used was 4.5 mM ARS in 100 mM of phosphate buffer pH 7. The cryogels were in contact with the dye for 4 hours and the solution was exchanged after 30, 60 and 150 minutes under agitation (20 rpm) using the Stuart rotator SB2 from Bibby Scientific Limited (Staffordshire, UK) at RT. After dye adsorption, the cryogels were washed several times with 100 mM of phosphate buffer, pH 7 until the solution was uncolored. Dye was eluted with 500 mM sorbitol in the same phosphate buffer until no remaining dye could be detected. A nonfunctionalized cryogel was used as negative control. The measurement was monitored by SPECTRAmax Plus

384 spectrophotometer microplate reader from Molecular Devices (Sunnyvale, CA, USA) at λ = 500 nm. This assays were equally applied for Boric acid gel in 96-well membrane plates 0.45 µm using MultiScreen™ Vacuum Manifold 96-well system for filtering dye solutions, as well as wash and elution steps and a ratio of 1:10 Boric Acid gel:dye solutions was used.

2.4.2 IgG binding assay

After functionalization, the cryogels were also analyzed by IgG adsorption regarding ligand immobilization efficiency. Initial screening was performed using 0.5 mL cryogels. The assays were operated manually, by batch adsorption, with a pipette and the syringe piston was manually pressurized after each step. The sequence of adsorption experiments after hydration and equilibration steps was as follow: (i) loading 500 µL of 2 g/L polyclonal IgG, (ii) two steps of washing (2 mL and 1 mL, respectively); (iii) two elution steps (2 mL and 1 mL) and finally (iv) two steps of reequilibration of 1 mL each. 20 mM HEPES pH 8.5 was used for equilibrating and washing steps and 1.5 M tris (30 mM acetate pH 3 for MEP) for elution based on chromatographic performances described in the literature [3,12]. The samples from each step were collected and analyzed by SPECTRAmax Plus 384 spectrophotometer microplate reader at 280 nm. The functionalized cryogels with higher adsorptions were selected for further scaling-up until to 1.6 mL cryogels in order to be used into chromatographic columns. The following selected reactions were performed:

- ED + 3FBPA at pH 8.0
- NH3 + CD + 3CPBA at pH 8.0
- ED + CD + 3CPBA at pH 8.0
- HMD + CD + 3CPBA at pH 8.0

- HMD + CD + 3CPBA at pH 4.5
- HMD + BDGE + MPBA

These 1.6 mL cryogels functionalized using the conditions developed to 0.5 mL cryogels were fitted in Tricorn™ 10/20 columns (i.d. 10 mm). The process was operated by Akta purifier with on line data acquisition under the same conditions as batch adsorptions using from 0.5 to 3 mL/min of flow rate.

3. Results and discussion

3.1 Adsorption studies on commercial boronate columns

Boric acid gel and Agarose P6XL are commercial boronate matrices used for capturing *cis*-diol compounds. These two matrices were used in order to investigate the adsorbed behavior of polyclonal IgG, since antibodies are mostly glycosylated, and the main impurities encountered in cell culture supernatant from IgG production, HSA and human insulin. Different binding conditions were studied by varying the pH and buffer type as shown in Table 1. The first results with phosphate buffer at Figure 2 show that at pHs 7 and 7.5 more than 95% of IgG loaded into the column. IgG adsorption was similar for both boric acid gel (Figure 2 A) and agarose (Figure 2 B). Regarding the impurities, insulin was highly retained (more than 70%) in all conditions with PBS. On the other hand HSA was lower than 40%, with higher retentions (around 25% higher) with boric acid gel matrix.

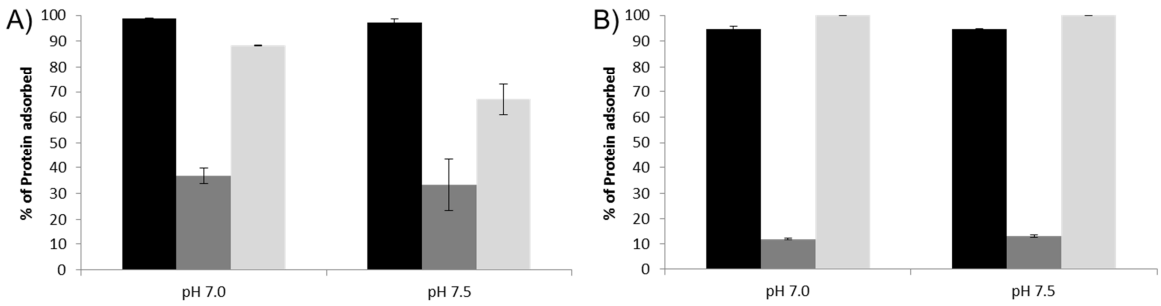

Fig. 2: Polyclonal IgG (black bars), HSA (darker gray bars) and human insulin (lighter bars) adsoption in Boric Acid gel (A) and Agarose P6XL (B) supports at pHs 7.0 and 7.5 using PBS buffer. Adsorption values were calculated based on peak areas obtained after elution divided per all peak areas of the chromatogram.

HEPES was the second binding buffer used at a pH range varying from 7.5 to 8.5. According to the profile obtained at a lower pH, higher IgG adsorption was observed as well as for the impurities HSA and insulin (Figure 3) leading to a poor selectivity for both matrices. HEPES buffer has been described to enhancer *cis*-diol esterification with PBA [18,33]. Indeed, Azevedo et al. [3] selected 20 mM HEPES pH 8.5 as standard conditions for the IgG adsorption studies since it provided the best efficiency for ProSep®-PB matrix. A similar behavior was observed with HEPES buffer and PBS at pH 7.5 for both matrices agarose and methacrylate, but a lower adsorption of IgG at pH 8.5 was observed [3]. On the other hand a better selectivity was obtained at pH 8.5 since HSA and insulin were less than 10% adsorbed. This value was much lower compared to the literature, around 70% of HSA adsorption was obtained under the same binding condition when ProSep®-PB matrix was applied [3].

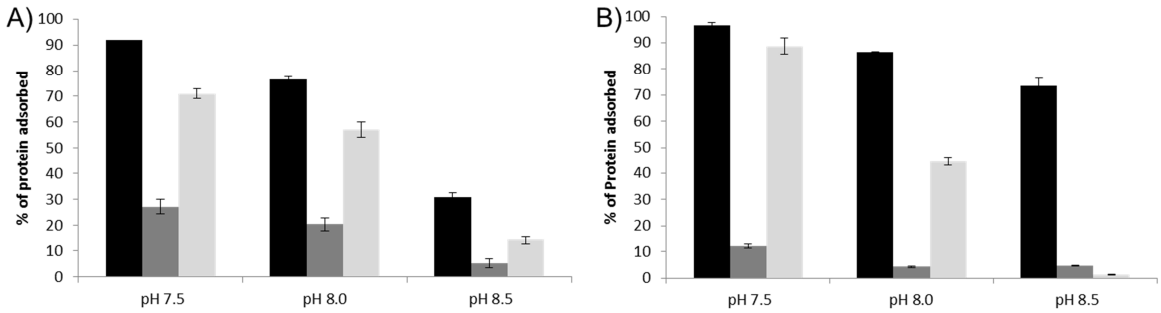

Fig. 3: Polyclonal IgG (black bars), HSA (darker gray bars) and human insulin (lighter bars) adsoption in Boric Acid gel (A) and Agarose P6XL (B) supports at pHs 7.5, 8.0 and 8.5 using 20 mM HEPES buffer supplemented with 150 mM NaCl. Adsorption values were calculated based on peak areas obtained after elution divided per all peak areas of the chromatogram.

IgG adsorption was lower than 80% under all conditions applied with borate buffer. With Boric Acid gel was observed a similar profile to that obtained when HEPES buffer was used: the lower the pH, the higher the adsorption of IgG and insulin. On the other hand, agarose provided the best IgG adsorption at pH 8.5 (~80%) when borate buffer was used and high selectivity was obtained since both HSA and insulin were not retained under this condition. An abrupt drop on adsorption was obtained when pH 9 was applied. The lower retention obtained with this buffer could be probably due to the washing of proteins by borate buffer. Studies with this buffer showed its ability to wash glycosylated proteins from anion exchange chromatography at pH 8 [34]. In addition, borate buffer may also compete with proteins for binding to the matrix which could explain the lower adsorption obtained at pH 8.5 and 9 when most of PBA are at tetrahedral conformation.

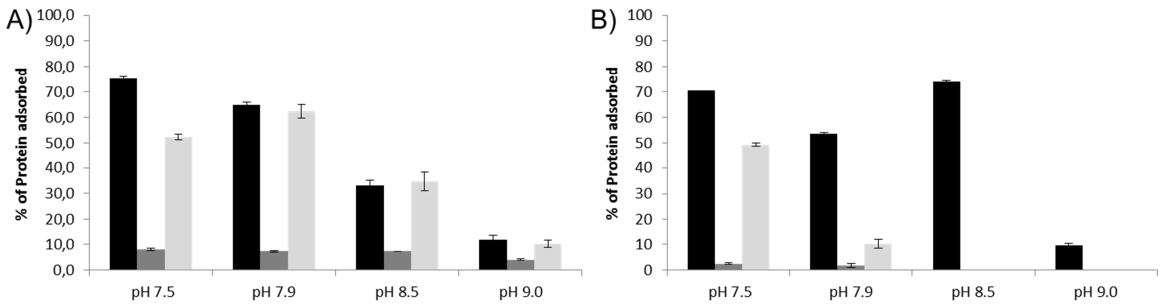

Fig. 4: Polyclonal IgG (black bars), HSA (darker gray bars) and human insulin (lighter bars) adsoption in Boric Acid gel (A) and Agarose P6XL (B) supports at pHs 7.5, 7.9, 8.5 and 9.0 using 50 mM borate buffer supplemented with 150 mM NaCl. Adsorption values were calculated based on peak areas obtained after elution divided per all peak areas of the chromatogram.

In general, at neutral pH higher IgG adsorption was observed but also superior retention of impurities for all buffers applied. The higher retention of IgG at neutral pH has already been reported in the literature when magnetic particles functionalized with PBA were used [12]. At pHs 8.5 and 9 lower IgG adsorption was obtained although PBA is at tetrahedral conformation. Studies referred in the literature with this polyclonal IgG [3,12] evidenced the presence of proteins pI ranging from neutral to 9 and the possible presence of proteins negatively charged probably shielded the interactions by repulsion with negatively charged PBA at tetrahedral configuration. The presence of 150 mM NaCl at binding conditions is probably not enough to shield this electrostatic repulsion. Studies by Zhang et al. [17] using boronate chromatography at pH 8.7 for capturing recombinant humanized monoclonal antibody, showed that 200 mM NaCl was the optimal concentration to shield the electrostatic repulsion observed at lower NaCl concentrations. This observation was confirmed by the adsorption studies performed in this work at chapter III using neutral and acidic glycosylated proteins in the same chromatography at pH 9.

A comparison between the two supports used, showed that Agarose P6XL provided higher selectivity than Boric Acid gel, especially at higher pHs when HEPES and borate buffers were used. Moreover, agarose has presented slightly higher dynamic binding capacity (DBC) at 10% of breakthrough (q_{10}) when IgG was loaded at pH 8.5 using HEPES buffer as binding condition supplemented either with 150 mM or 300 mM NaCl (Table 2). This fact could be probably related to PBA density since Agarose P6XL has around 38% higher ligand density than Boric Acid gel (14.26 ± 0.47 μmol/mL and 8.88 ± 0.19 μmol/mL, respectively) according to the studies performed with ARS (see section 2.4.1). Even though, agarose DBC is relatively low when compare with results referred in the literature with, ProSep®-PB (10.4 mg/mL) [3] and *de novo* design ligand mimicking the protein A (more than 50 mg/mL) [35]. However, the mimetic ligand showed a low selectivity with almost the same DBC for both IgG and HSA. According to the results showed in Table 2, higher concentrations of NaCl decreased the binding capacity of IgG for both matrices, meaning that probably electrostatic repulsions are not the only motive for the lower IgG binding at higher pH.

Table 2: Dynamic binding capacity at 10% of breakthrough of Boric Acid gel and Agararose P6XL with polyclonal IgG at pH 8.5.

	q_{10} (mg IgG/mL matrix)	
Buffer	20 mM HEPES pH 8.5	
NaCl supplementation	150 mM	300 mM
Boric Acid gel	2.34 ± 0.45	1.76 ± 0.11
Agarose P6XL	3.16 ± 0.06	2.58 ± 0.53

3.2 Cryogels functionalization with PBA

Cryogels functionalized with PBA have been widely studied in the last years [26,27,36]. These supports present several characteristics such as macroporosity, low cost

production and easy handling that make them very advantageous for industrial applications, particularly for large biomolecules as cells, RNA and proteins separations [26]. Thus, cryogels functionalized with PBA could be a useful tool for glycosylated IgG purification and to further integrate clarification and capturing steps by retention of IgG into the PBA cryogel whereas the cells would pass through the column. In order to explore this concept, several reactions were performed in order to immobilize PBA on the cryogel with functional epoxy groups. Different PBA derivatives were tested and compared by determining PBA ligand density by ARS analysis and polyclonal IgG retention (Figure 5). The ratio of IgG adsorption per PBA immobilized was also analyzed as it can be seen in Figures 5 A to D. Cryogels without PBA were only analyzed by IgG adsorption (Figures 5 E and F). The IgG binding studies were first performed at batch conditions as described in section 2.4.2.

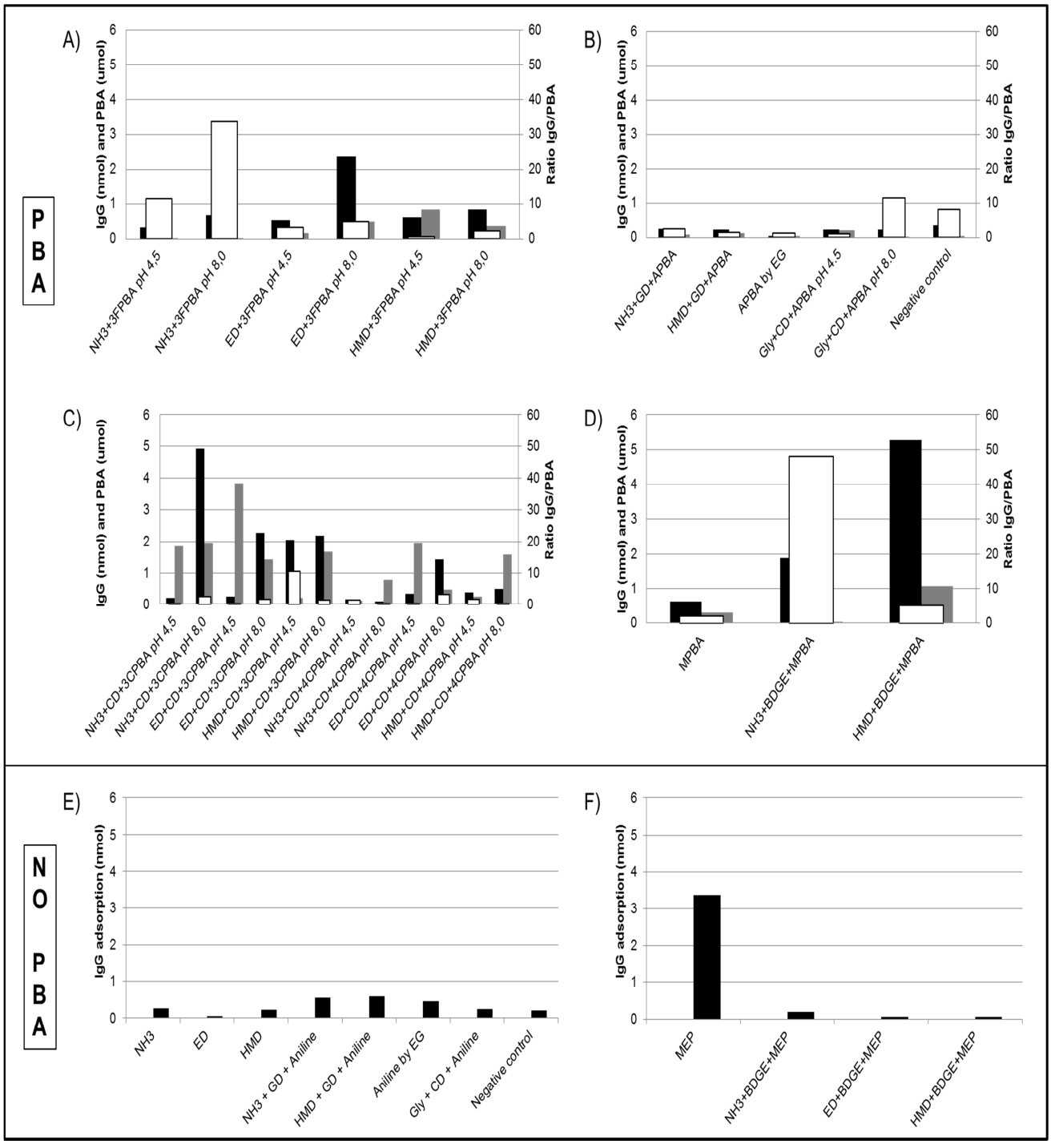

Fig. 5: Concentration of IgG adsorbed (black bars), PBA ligand density by ARS analysis (grey bars) and ratio IgG per PBA (white bars) from cryogels immobilized with different PBA derivatives in several conditions (A – FPBA, B – APBA, C – 3- and 4-CPBA and D – MPBA), with ammonium or space-arms (E) or with MEP at different conditions (F).

PBA derivative APBA showed the lowest IgG adsorption values as well as the lowest ligand density in comparison to the other PBA derivatives tested with maximum adsorption of 0.5 nmol of IgG (0.15 mg of IgG/mL of cryogel), as it can be seen in Figures 5 A-F. The highest adsorptions (around 5 nmol of IgG, i.e., 1.5 mg of IgG/mL of cryogel) were obtained with 3-CPBA and MPBA (Figures 5 C and D, respectively). The results obtained for IgG adsorption onto the functionalized cryogels by different PBA derivatives and conditions have not showed a particular trend that could serve as a guideline to choose a PBA derivative, ligand density or a certain condition to get higher IgG adsorptions. For instance, the highest adsorptions obtained with formyl and 3-carboxyl derivatives were performed at pH 8, although this was not a rule for all reactions at pH 8. Besides, the higher adsorptions were not always obtained with the cryogels with higher ligand densities. MPBA derivative showed the highest IgG adsorptions when ammonium or HMD were used to activate the epoxy groups of cryogel (Figure 5 D), but no retention was observed without activation of the epoxy groups by amination. The opposite behavior was observed with MEP derivative (Figure 5 F) with higher adsorption around 3.2 nmol of IgG (0.96 mg of IgG/mL of cryogel) when the epoxy groups were not activated. This ligand has the same functional group as MPBA (mercapto) and was referred in the literature as able to capture monoclonal antibodies, with IgG adsorption of 25 mg/mL of support [37,38]. MEP is a hydrophobic charge induction chromatography which employs high heterocyclic ligand densities avoiding the need of high salt concentration. When pH is below its pKa (4.8), this ligand becomes positively charged what is explored for proteins elution by positive charge repulsion [16]. In this work, MEP provided higher adsorption (3.2 nmol of IgG) than most of immobilizations with PBA except than MPBA immobilized with the space-arm HMD and 3CPBA at pH 8 with the ammonium. These both immobilizations (MPBA and 3CPBA) provided the highest adsorptions values reaching around 5 nmol of IgG (75% of IgG loaded). The best results obtained in this work are still low compared to

other types of boronate chromatography supports [3,12,17]. Anyway the best functionalized cryogels giving the highest IgG adsorption were thus selected for the experiments using a continuous column bed chromatography (see selected reactions at section 2.4.2) using cryogels with larger volume (1.6 mL).The experiments performed using a continuous mode lead to no adsorption of IgG onto the selected supports even when the sample was loading at low flow-rate of 0.5 mL/min. Although similar ligand densities were obtained for these larger supports, this seems to not be enough for capturing the IgG using this column system. Direct immobilization of PBA onto cryogels by copolymerization during support production has been also referred in the literature [26,27]. Srivastava et al. [27] have recently reported the production of copolymerized poly (hydroxyethylmetacrylate) cryogel with vinylphenylboronic acid. According to these authors, the copolymerization could not provide freely PBA at the surface which could hinder recognition of the cells to the ligand although not affecting RNA and glycosylated proteins recognition. This concept was demonstrated by the successfully isolation of RNA from crude of *E. coli* lysate. This approach can be a potential alternative for IgG separation by integrating clarification and capture steps. Besides with this methodology a higher ligand density could be obtained increasing column binding capacity although monolith supports are known for providing lower capacities compared to beads [23].

4. Conclusions

Phenylboronate chromatography is usually performed at alkaline pH in order to retain *cis*-diol compounds in its tetrahedral conformation promoting the interactions via this group with high efficiency, although PBA multimodal behavior allows different types of interactions with the target molecules also at a tetrahedral conformation [17]. This behavior can probably explain the higher IgG adsorption observed at neutral conditions (> 95%),

although the low selectivity (70% or higher of insulin retention) obtained in the experimental conditions tested with commercial matrices Agarose P6XL and Boric Acid gel. Relatively to the two tested matrices, Agarose P6XL had a better performance than Boric Acid gel regarding selectivity and capacity, being more appropriate for IgG capture by liquid boronate chromatography.

Concerning cryogels studies, no relation was obtained between IgG adsorption and ligand density. Higher IgG retentions (mostly around 0.6 mg of IgG/mL of cryogel) were obtained with formyl and carboxy PBA derivatives when immobilized at pH 8. With MPBA with HMD space-arm good IgG retentions were also observed (around 1.5 mg of IgG/mL of cryogel). The results obtained in this work have shown the potential of PBA chromatography for IgG specifically adsorption, although much more work is need in terms of optimization of the binding conditions in order to obtain high yields without losing selectivity. Another important aspect is the ligand density and the production of cryogel copolymerized with PBA could probably be used in order to increase ligand density and thus column capacity.

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CHAPTER VI

CONCLUSIONS AND FUTURE TRENDS

Conclusions and Future Trends

1. Conclusions

1.1 Fundamental studies

Most of the applications of phenylboronate chromatography (PBC) referred in the literature have explored the esterification of PBA with *cis*-diol as main interaction although the mixed-mode behavior has already been discussed for more than 20 years [1]. As presented in the first part of this work, multimodal chromatography can bring higher efficiency and selectivity over single modal chromatography with the correct exploitation of the multimodal behavior. Indeed, several recent applications of PBC on *cis*-diol biomolecules separations have pointed out that this multimodal chromatography is a powerful tool for biomolecules purification. In addition, the ligand chemical stability and its low cost compared to other affinity ligands, especially the biological ones, makes this chromatography a potential technique for applications at industrial level.

The multimodal behavior of PBC was evaluated by performing adsorption studies using a protein library containing both glycosylated and nonglycosylated proteins with a wide range of molecular weights and pI values. According to the literature, PBA interacts with proteins via several modes of interaction, including affinity *cis*-diol, electrostatics, charge transfer and hydrophobic interactions. The results obtained in this work have shown that PBC was able to adsorb all the proteins tested but each class of protein provided a specific adsorption pattern regarding the pH range studied from 4 to 9. Acidic proteins provided adsorption at lower pHs, mostly below 7; neutral proteins were highly adsorbed between pH 5 and 8 whereas basic proteins were retained at basic conditions with higher adsorptions above pH 7. Regarding glycosylation, basic glycosylated protein

(RNase B) was highly adsorbed at pHs above 7, but acidic and neutral glycosylated proteins (invertase, amyloglucosidase and cellulase) were retained only in the presence of 200 mM NaCl under these conditions. Electrostatic interactions by negatively charged PBA at tetrahedral conformation are clearly playing a role at high pHs, binding positively charged basic nonglycosylated proteins (RNase A) and repelling affinity *cis*-diol interactions of negatively charged acidic and neutral glycosylated proteins at alkaline pHs. In addition, affinity *cis*-diol interactions were shown to be NaCl independent.

Retention of proteins by the trigonal conformation of PBA was also investigated and it was observed that pepsin, carbonic anhydrase and cellulase were differently mitigated by citrate, a hard Lewis bases, emphasizing that probably Lewis acid-base interactions by charge transfer could play a significant role under these conditions, although the same behavior was not observed with other buffers known as Lewis bases. According to some protein surface property analysis, the different effect of citrate on proteins adsorption could be related to the presence of hydrophobic patches near negatively charged amino acids of carbonic anhydrase which could strengthen protein PBA interaction. Further studies performed in this thesis showed that the interaction between the negatively charged citrate and PBA could also help on mitigating interaction of proteins. For instance, pepsin is negatively charged under acidic pH but carbonic anhydrase and cellulase are positively charged which allowed the selective binding.

Hydrophobic interactions were also investigated and the addition of 20% ethanol in the binding condition led to a decrease of around 25% on proteins adsorption. Further investigations were carried out using phenylsepharose chromatography by analyzing proteins hydrophobic patches by fluorescence. For RNase A and B a relation between proteins hydrophobic patches and retention onto phenylsepharose matrix were observed at pH 4 but no binding was obtained with PBC under the same conditions,. The boronate moiety increases the polarity which probably shields such effect. No relation between

hydrophobic patches and phenylsepharose was observed with cellulase and pepsin. These proteins provided retention at pHs 4 and 9 whereas nothing was obtained at fluorescence measurements. Analysis by SAP maps showed that pepsin has higher number of hydrophobic patches than cellulase, although the higher number of negatively charged amino acids present on pepsin probably dominated over the hydrophobic surface which partially can explain the higher mitigation of pepsin adsorption by citrate at PBC. Carbonic anhydrase had no retention on phenylsepharose chromatography although the presence of hydrophobic patches was observed by SAP maps and fluorescence analysis. This fact could reinforce the hypothesis that the hydrophobic patches near negatively charged residues may stabilize the interaction.

Displacer gradient was the most effective strategy resulting in the highest recovery yields and best selectivity. Tris and citrate buffers were able to selectively separate glycoproteins and neutral/acidic proteins, respectively. Regarding glycosylated proteins, usual modifiers were used in these studies and tris, sorbitol and fructose led to recoveries about 100% although only with tris a good selectivity was obtained.

Comparison of two PBC matrices was also performed by using ProSep®-PB and Agarose P6XL, controlled-pore glass and agarose base, respectively. The agarose matrix provided a shorter pH range regarding the adsorption of the tested proteins. This behavior could probably account for a higher selectivity of this matrix which was confirmed by adsorption studies with both matrices at different pHs. The glass matrix provided better performance although it has lower ligand density compared to agarose beads (8.76 ± 1.83 µmol/mL and14.26 ± 0.47 µmol/mL, respectively). This is probably related to the matrix characteristics. Controlled-pore glass is a silica based matrix and due to hydroxyls of silica above pH 6 the matrix is negatively charged [2] which could be responsible for protein stabilization by electrostatic interactions. In addition, this matrix has

larger pores what could decrease the steric hindrance and consequently increase ligand availability. CPG is also a hydrophilic matrix widely applied for glycoproteins separation [3].

PBC proved to be a powerful technique for separation of proteins. By rationally exploiting phenylboronate interactions, highly selective windows of separation could easily be achieved for the fractionation of complex biological mixtures. In addition, this work can give insights in many of the applications already described for the PBA ligand.

1.2 Studies on IgG adsorption

Commercial phenylboronate matrices based on methacrylate and agarose were used to specifically bind a polyclonal antibody regarding a mixture of contaminants proteins and to determine the best binding conditions in order to obtain high recovery and selectivity. This chromatography has proven to be able to capture and purify polyclonal antibodies. In general, higher protein retentions were observed at neutral pHs although a lower selectivity was obtained as human insulin and HSA were also retained at this conditions. The association constants of *cis*-diol esterification are usually lower under neutral pH and other type of interactions are thus responsible at this pH for a higher retention of nonglycosylated protein as insulin.

Immobilization of phenylboronic acid onto cryogels monolithic supports with functional epoxy group were also carried out in order to further purify monoclonal antibodies by integrating clarification and capture steps. The highest IgG adsorptions (1.5 mg of IgG/mL of cryogel) were obtained with MPBA at batch conditions.

2. Future Challenges

This work identified two distinct pH ranges in which phenylboronate can provide distinct interactions with proteins. In addition to the highly exploited *cis*-diol affinity interaction that is dominant at alkaline pH, charge transfer interactions can be exploited for the separation of biomolecules at lower pH.

The effect of phenyl moiety is still not completely understood and different type of interactions, such as hydrophobic, cation-π or π-π can occur at all pH ranges. Further studies should be carried on in order to fully understand and identify the type of interactions involved regarding protein type and binding conditions. Adsorption studies could be performed with different modifiers. Dougherty, D.A. [4], described the importance of cation-π interactions and have suggested that amino moieties of some amino acids as arginine and lysine can perform this specific interaction, on the other hand glutamine and asparagine can only make polar-π interaction. In addition, Mg^{+2}, Li^{+} and K^{+} are cationic agents described in the literature with ability to perform cation-π interactions [1,4]. Regarding π-π interaction, another aromatic reagent could be used in the system, phenylalanine has already been used in order to displace nonspecific interactions between PBA and heavy chain of recombinant humanized mAb, but no elution was obtained [5]. Even though, a different effect could be obtained using this modifier at binding condition since the interaction could not be strong enough to disrupt but it could avoid further interactions.

The hydrogen bond effect can be further investigated by mitigating this interaction on proteins adsorption as the studies performed in the Chapter III. Urea has been reported as able to disrupt such interaction between thiourea and PBA [6] and it could be used in adsorption studies under acidic and alkaline pHs in order to study this interaction by different classes of proteins from the protein library.

Studies on protein binding sites could also help in understanding the predominance of interactions in different classes of proteins under different conditions. Saturation transfer difference nuclear magnetic resonance (STD-NMR) spectroscopy has been reported as an easy and fast tool for characterization of protein binding sites as well as ligand screening [7] and could be perfectly applied for the purposes of this work.

Concerning to the elution studies performed in the Chapter IV, the α-hydroxyacid citrate provided certain selectivity when applied on acidic and neutral proteins separations under acidic conditions. Other α-hydroxyacids could be explored in order to enhance the selectivity of this separation. Malic and salicylic acids are some examples of this class of acid which could be applied in this study.

Adsorption studies of IgG and impurities (human insulin and HAS) with ProSep®-PB and Agarose P6XL matrices should be designed to take advantage of the different type of interactions of these contaminant proteins with PBA at different binding conditions, in order to obtain a selective adsorption. The results obtained in this work have shown the potential of PBA chromatography for IgG specifically adsorption, although much more work is needed in terms of optimization of the binding conditions in order to obtain high yields without losing selectivity.

Direct immobilization of PBA onto cryogels by copolymerization during support production could probably be used for obtaining a higher ligand density and better performance. The production of this support has already been described in the literature using vinylpheylboronic acid as the derived of PBA for direct reactions with hydroxyethyl methacrylate monomers [8] but applications for IgG capture have not been described. This approach can be a potential alternative for IgG recovery facilitating process integration by combining IgG clarification and capture steps. Besides with this methodology a higher ligand density could be obtained increasing column binding capacity.

Other potential applications for phenylboronate chromatography are related to separation of different classes of proteins for proteomics studies. This unique interaction of PBC could also be explored for separation of proteins isoforms based on the double peaks obtained at results from pH gradient for some proteins which could also indicate the separation of isoforms.

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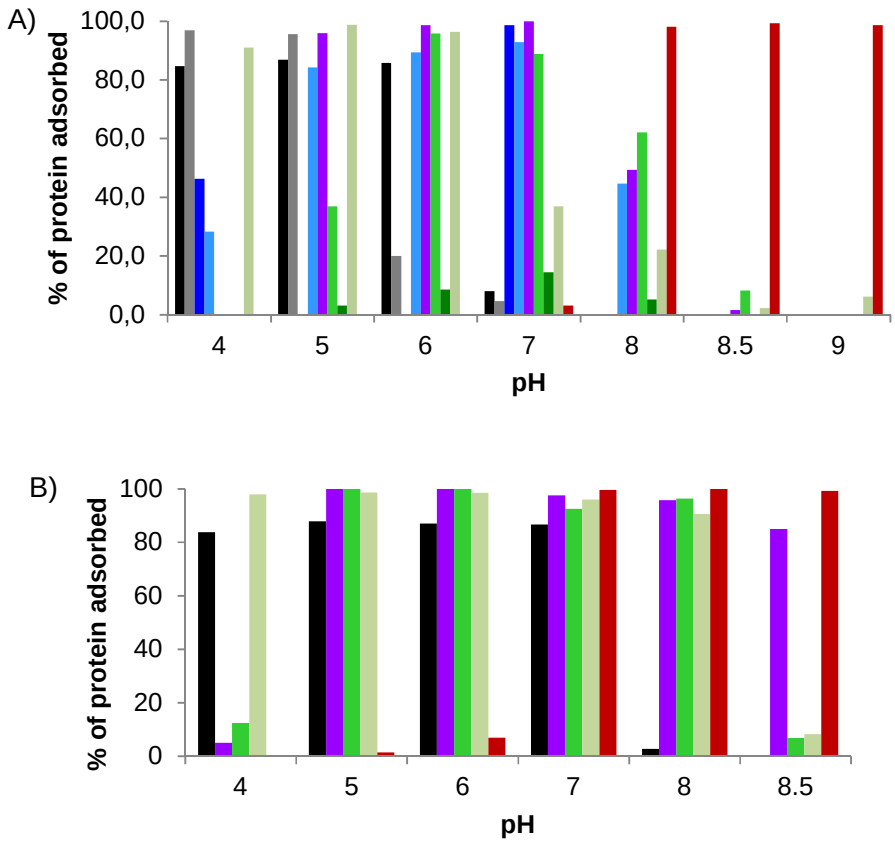
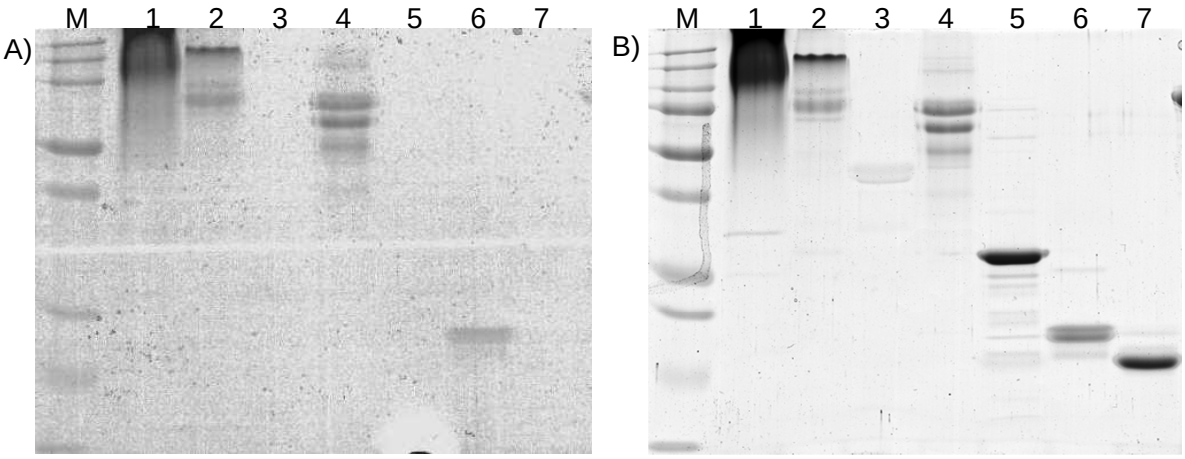
CHAPTER VII

APPENDICES

Appendix A: Building the protein library



A1: Percentage of adsorption of proteins at Agarose P6XL (A) and ProSep®-PB (B) matrices (2 mL column bed) in a pH range from 4 to 9. The experiments for building a protein library was performed according methods presented in the section 2.2.1 of chapter III with application of same buffers and conditions. The following proteins were used in these studies: acidic nonglycosylated pepsin pI 2.6 (black bars), acidic glycosylated amyloglycosidase pI 3.6 (gray bars), acidic nonglycosylated insulin pI 5.2 (dark blue bars), acidic nonglycosylated lipoxidase pI 5.7 (light green bars), neutral nonglycosylated conalbumin pI 6.1 (purple bars), neutral nonglycosylated carbonic anhydrase pI 6.6 (fluorescent green bars), neutral glycosylated myoglobin pI 7.2 (dark green bars), neutral glycosylated cellulase pI 7.7 (light green bars) and basic cytochrome C pI 10.3 (red bars).



A2: SDS-Page (section 2.4 – chapter IV) of proteins selected for the protein library used in this work: invertase (lane 1), amyloglucosidase (lane 2), pepsin (lane 3), cellulase (lane 4), carbonic anhydrase (lane 5), RNase B (lane 6), RNase A (lane 7) and marker (lane M). The same gel were first stained with glycoprotein detection kit from Sigma-Aldrich (code: GLYCO-PRO) for reveal the glycoproteins of the library (A) and after it was stained with comassie brillant blue (B) to reveal all proteins.

Appendix B: Supplier specifications of commercial supports used in this work

B1 ProSep®-PB from EMD Millipore

Specifications	
Matrix	Porous glass with particle size in the range ≥ 74 ≤ 125 microns
Working pH Range	pH 2.5 – 8.5
Leachables	0.3ppm Si at pH7, decreasing at lower pH (0.01 ppm at pH 1.5). ProSep-PB media is stable between pH1 and pH9.
Pressure	Incompressible matrix with linear pressure/flow rate characteristics. Maximum operating pressure > 3000 psi (200 bar)
Shelf Life	1 year
Life	ProSep-PB media is stable over repeated operational cycles provided that proper cleaning protocols are observed.

Data source: the full supplier information can be found in the following link: http://www.millipore.com/publications.nsf/a73664f9f981af8c852569b9005b4eee/7388d231c850c11485256db4004472d7/$FILE/DS1055EN00.pdf

B2 Agarose P6XL from ProMetic Biosciences

Adsorbent	Aminophenylboronate A6XL	Aminophenylboronate P6XL
Support matrix:	6% cross-linked agarose	6% cross-linked monodisperse agarose
Bead size:	45 - 165 microns	100 microns (95% between 76-141 microns)
Exclusion limit:	4 x 10^6 Daltons	4 x 10^6 Daltons
Temperature Range:	2 – 120°C	2 - 120°C
pH Range:	3-11 (continuous) 2-14 (intermittent)	3-11 (continuous) 2-14 (intermittent)
Max. Pressure:	15 psi (1 bar)	15 psi (1 bar
Storage:	2-30°C in solution of 20% Ethanol	2-30°C in solution of 20% Ethanol
Cleaning/ Pyrogen Removal	20mM acetic acid/1M NaOH	20mM acetic acid/1M NaOH
Chemical Compatibility:	Compatible with most buffers and common additives.	Compatible with most buffers and common additives
Sterilization:	1M NaOH (2 hours); autoclave 30 min., 120°C, pH 7.0	1M NaOH (2 hours); autoclave 30 min., 120°C, pH 7.0

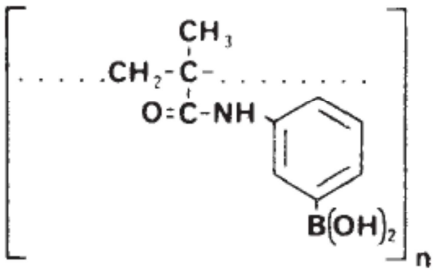
Data source: Technical User Guide: Aminophenylboronate A6XL 0355 and Aminophenylboronate P6XL 3355. ProMetic Biosciences, pg. 3. Printed catalog obtained with product. Supplier website: www.prometicbioscience.com

B3 Boric Acid gel from Sigma-Aldrich

Description and Characteristics

Boric Acid Gel is a cross-linked polymer insoluble in water and all organic solvents. It is prepared by the cross-linking copolymerization of dihydroxyborylanilino-substituted methacrylic acid with 1,4-butanediol dimethacrylate.

The gel is swollen in distilled water, then activated with 0.5*N* HCl. It is then washed to neutral pH and vacuum-dried. Thus, the gel is supplied "activated," as a nearly free-flowing granulate.



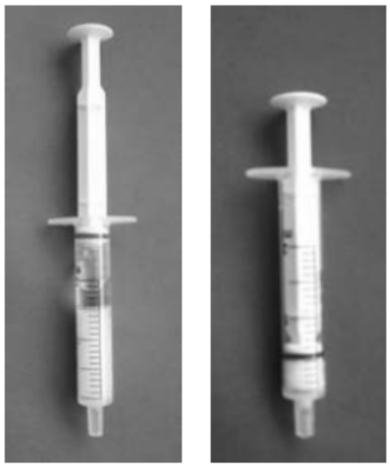
Appearance	nearly dry, off-white granules
Boron content	1.4% (dry)
Packing volume	ca. 0.6g/mL
Degree of swelling	ca. 80% (i.e., final increase in volume ca. 20%)
Bead size	0.1-0.4mm
Ribose-binding capacity	approx. 0.01mmol/mL (The Boric Acid Gel offered by Aldrich has been shown to possess a binding capacity four times that reported in the literature.

Data source: the full supplier information can be found in the following link: http://www.sigmaaldrich.com/etc/medialib/docs/Aldrich/Bulletin/al_techbull_al102.Par.0001.File.tmp/al_techbull_al102.pdf

B4 Cryogel BasicE from Protista

Description of Cryogel BasicE plugs

Bed volume:.....1 or 5 ml
Matrix:.....polyacrylamide
Pore size:.....10-100 µm
Pore volume:.....≈ 90%
Functional group:.....epoxy (30 µmol/ml)
Chemical stability: …all commonly used buffers
Recommended working pH.....3-10
Compressibility of the matrix:.....4- to 5- fold



Cryogel BasicE: hydrated and compressed with a plunger plugs (right) inside a plastic column.

Data source: Cryogel BasicE product information. Protista, pg. 2-3. Printed information sheets obtained with product. Supplier website: www.protista.se