TÉCNICO LISBOA

iBB
Institute for Bioengineering and Biosciences

Extended Abstract

Purification of antibody fragments by phenylboronate chromatography using a microfluidic device

Miguel Ambrósio

Antibodies are glycoproteins used in a wide range of fields because of their affinity and specificity towards specific antigens. Alternatives to full-sized antibodies have also been developed, as antigen-binding fragments (Fabs), which are today the most widely used. Despite their increasing popularity, there is no preferable method to purify these antibody derivates, since there is no universal affinity ligand. In the last years, phenylboronate has been successfully employed to capture monoclonal antibodies, due to its affinity to cis-diol groups, and, more recently, for binding to kappa Fab fragments.

In this work, the digestion protocol of human antibodies was initially optimized, with an increase of 41% in the antibody digestion yield obtained. Then, the possibility of using aminophenylboronate for purification of kappa and lambda classes of Fabs was thoroughly investigated. A microfluidic platform was used to screen different binding and elution conditions in a high throughput manner, followed by validation at macroscale. Several impurities related with the manufacturing process of Fabs were also studied. Overall, below ligand pKa, affinity binding, enhanced by charge transfer interactions, was the driving force for the adsorption process. Optimal binding of Fabs occurred at pH 5 and 7 for intermediate and lower concentrations of salt, respectively. At pH 9.2, electrostatic repulsion supressed affinity interactions. Due to the presence of different isotypes and subclasses and also due to polyclonal nature of the feed, Fabs were found in both flow-through (FT) and elution (E) fractions. Further studies would be required in a case-by-case approach to assess the behaviour of this ligand towards a monoclonal Fab.

Introduction

Antibodies are glycoproteins, naturally found in the human body, that can recognize and bind to a given antigen with a high degree of affinity and specificity. Because of these properties, immunoglobulins have been used for a wide range of applications, namely in biotechnology, pharmaceutical and medical fields[1]. However, their utilization has some limitations, including restricted penetration and homogenous distribution in target tissues and triggering of secondary immune reactions. They also present a prolonged half-life time, which can be a limiting factor for some applications. All these constrains are caused by the large molecular height of full-sized antibodies[2]. In the past decades, development of alternatives to monoclonal antibodies has gained relevance, particularly smaller proteins with enhanced properties[2]. Antigen-binding fragments, or Fabs, were the first immunoglobulin fragment-based technology to be developed and it is the most widely used, offering a set of unique properties, including reduced half-life times and enhanced tissue penetration[3]. However, in opposition to mAbs, there is no standard method of choice to capture Fabs, once there is no universal solution. Instead, a set of alternatives can be used, including affinity (e.g. protein L) and non-affinity methods (e.g. hydrophobic and ion-exchange chromatography), but all of them present limitations and drawbacks[4]. Therefore, new methods for Fab purification need to be developed, ideally for all classes of fragments and independent from the manufacturing method being used. For the past years, phenylboronate multimodal ligand has been successfully employed to capture monoclonal antibodies, due to its affinity to cis-diol groups[5,6]. Recently, Nascimento and co-workers also showed the possibility of using this ligand to efficiently capture kappa Fab fragments[7].

The first aim of this work was to screen and optimize different variables in the papain-based antibody digestion protocol, in order to obtain a more efficient process. The variables studied included reaction time and volume, enzyme and substrate concentrations, temperature, operational pH and buffer type. The establishment of an efficient digestion protocol allowed cost and time savings, and a proper characterization of the impact of each variable on the digestion process. The second and main aim of this work was to study the possibility of using aminophenylboronate as a universal ligand for purification of kappa and lambda classes of Fabs. For this, different chromatographic conditions were screened in a high throughput manner, using the microfluidic device previously developed by Pinto et al.[8] The screening included a set of different pH and salt concentration for binding, where also several impurities related with the fragments manufacturing process were tested - undigested antibody, Fc fragments and proteins from CHO-S supernatant - and several agents for elution, such as tris, D-sorbitol, magnesium chloride, guanidine hydrochloride, urea and arginine. These studies were complemented with the validation of results at macroscale, also allowing the extraction of additional information, as the proportion of protein bound to the column versus total protein injected and results comparison between different target molecules. The approach here used allowed the study of the interactions pattern leading to retention of target molecules in the column.

Materials and Methods

Chemicals and biologics

Human polyclonal antibody mixture, commercially named Gammanorm®, was obtained from Octapharma (Lachen, Switzerland), with a concentration of 165 mg/mL and 95% IgG (59% IgG1; 36% IgG2; 4,9% IgG3; 0,5% IgG4 and at maximum 82.5 µg/mL of IgGA). To label the IgG, BODIPY™ TMR NHS from Lumiprobe was dissolved in DMSO (Merck, Germany) and stored at minus 20ºC. CHO-S supernatant, grown in Freestyle™ CHO expression medium (Termo Fisher Scientific, USA), was kindly provided by Marta Carvalho from SCERG research group.

Tris (hydroxymethyl)aminomethane (Tris), sodium chloride, phosphate buffered saline (PBS), citric acid, D-sorbitol, EDTA, L-cysteine, iodoacetamide, papain, ammonium persulphate (APS), N,N,N,N-tetramethylethylenediamine (TEMED), glycine, sodium thiosulphate, sodium carbonate, glutaraldehyde, 30% acrylamide/bisacrylamide-solution, sodium carbonate, sodium bicarbonate, CHES, EPPS, urea and magnesium chloride were obtained from Sigma Aldrich (USA). SDS micropellets (sodiumdodecyl sulphate), 2x Laemmli sample buffer and Precision Plus Protein™ Dual Color Standards were acquired from BIO-RAD (USA), Blue safe from NZYtech (Portugal), isoelectric focusing (IEF) kit and marker from GE Healthcare (USA), trichloroacetic acid, dodecyl sulphate sodium salt (SDS), silver nitrate and formaldehyde from Merck and acetic acid from Fisher Chemical (USA). Formaldehyde solutions, sodium acetate 3-hydrate, disodium hydrogen phosphate and sodium dihydrogen phosphate were purchased from AppliChem PanReac. Also, L-arginine was acquired from ACROS organics (USA) and guanidine hydrochloride from Invitrogen (USA).

Chromatographic columns and devices

Pre-packed 5 mL and 1 mL HiTrap™ Protein L and HiTrap™ Protein A HP were obtained from GE Healthcare, PA Immuno-Detection sensor cartridge from Applied Biosystems (USA) and aminophenylboronate P6XL resin from Prometic Bioseparations (Canada). Amicon® Ultra-4 centrifugal filter units (MWCO 10, 30 and 100 kDa) used for diafiltration were purchased from Merck Millipore.

Labelling of target molecules

For the labelling of all target molecules, a protocol by São Pedro et al., 2019[9] was used, by adding the dye to a 20:1 molar ratio. After that, degree of labelling of each conjugate was calculated following Pinto et al., 2017[10].

Antibody digestion

The digestion protocol herein applied was based on the work of Andrew et al[11].

First, for screening of factors being relevant in the digestion process, digestion time was varied (0, 2, 4, 6, 8, 10, 12, 18, 24 hours) and, for each of these times, four different papain concentrations were used (0.2; 0.1; 0.05; 0.01 mg papain/mL). The remaining parameters (500 µL; 1 mg IgG/mL; PBS pH 7.4) were maintained as standard conditions in every experiment using this strategy, except when indicated. Then, for 6 hours of digestion and 0.1 mg papain/mL, different reaction volumes were tested (1, 2 and 5 mL), and for each of them, different IgG concentrations were used (1, 2, 5 and 10 mg IgG/mL). Also, different buffers combined with varying pH values were tested: 10mM phosphate buffer pH 6.5, 7 and 7.5, 10mM Tris buffer 7.5, 8, and 8.5 and 10mM Tris buffer 150mM NaCl pH 8, for 6 hours digestion, 1 mL final volume, 0.1 mg papain/mL and 1 mg IgG/mL. Finally, also using these conditions, different temperatures were tested (25, 37 and 40ºC). For the DOE approach, two parameters were changed at a time, including temperature (25, 33 or 40ºC), digestion time (1, 12.5 or 24 hours), papain concentration (0.01, 0.105 or 0.2 mg/mL) and Gammanorm® concentration (1, 5.5 or 10 mg/mL). In all experiments, EDTA and L-cysteine were kept to a final concentration of 0.015 M. To stop the reaction, a solution of 0.3 M of iodoacetamide was added to a final concentration of 0.03 M. The design of experiment (DOE), along with the treatment of multivariable data was performed using MODDE Go software, developed and commercialization by Umetrics. Data was adjusted to a multiple linear regression. D-optimal model was used for DOE.

Chromatographic runs

Chromagraphic experiments were performed using ÄKTA™ Purifier 10 system (GE Healthcare), which allowed conductivity, pH and UV absorbance at 280 nm continuous monitoring. All the data was acquired by the software Unicorn 5.1 and fractions were collected using a Fraction Collector Frac-950 from GE Healthcare.

Quantification of antibody digestion

After digestion, Fab fragments were indirectly quantified using an analytical protein A quantitative column, PA Immuno-Detection sensor cartridge, as performed by Nascimento and co-workers[7].

Downstream processing of Fabs

After labelling and enzymatic cleavage, digestion mixture was submitted to a downstream protocol developed by Nascimento et al., 2018[7], with the aim of obtaining purified kappa and lambda Fabs and Fc fragments. This consisted in a protein A, followed by a protein L affinity chromatography, using PBS pH 7.4 (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride) and 0.1 M citrate buffer pH 2.5, as binding and elution buffers, respectively. In both cases, flow-through and elution fractions were collected and neutralized by adding 2 M Tris pH 9. Diafiltration steps were performed to concentrate and purify the samples. For this, Amicon Ultra-4 centrifugal filter unites (MWCO of 30kDa) were used in all the cases, but for protein A elution fractions, a 100kDa MWCO membrane was previously used to exclude undigested IgG molecules. Protein concentration was determined by reading samples absorbance at 280 nm and using a calibration curve drawn with well-defined concentrations of Gammanorm®, ranging from 10 to 1000 mg/mL.

Fabrication of the microfluidic device

The microfluidic structure here used for the high throughput screening of chromatographic conditions was developed by Pinto and co-workers[10] and so the respective mold and manufacturing protocol of the device was used.

Packing and outline of microfluidic experiments

The protocol used was based on the work of Pinto and co-workers[10]. After packing and under an inverted fluorescence microscope (Olympus CKX41), coupled to a CCD colour camera (Olympus XC30), 30 µL of a solution containing the target molecule at

60 µg/mL is flowed through the microfluidic channel, allowing monitoring of labelled proteins, as they bind to the column. Images were acquired using an exposure time and a gain of 1 second and 0 dB, respectively. Fluorescence data was extracted from three representative beads using Image J software. All experiments were performed at a flow-rate of 15 µL/min. For elution studies, 50 mM phosphate no salt was used as adsorption buffer. OriginLab software was used to calculate Fmax for each adsorption condition, by adjusting results to an equation based on the Hill enzymatic kinetics model:

$$F = \frac{F_{max} \times (t)^n}{(t_{0.5})^n + (t)^n}$$

where F represents fluorescence of the beads, Fmax is the maximum rate of reaction, t is the time, t0.5 is the time that originates half of the Fmax and n is the dimensionless Hill coefficient. Origin software was also used to draw heatmaps for each target molecule. To compare elution results, slopes for the first 15 seconds were determined.

Validation of the results at macroscale

A 1 mL column was manually packed with aminophenylboronate P6XL resin. All buffers were flowed into the column at a flow-rate of 1 mL/min. An equilibration step was always performed prior to injection, consisting in the addition of 10 column volumes (CV) of adsorption buffer. For all chromatographic runs, 200 µL solution containing 1 mg/mL of target molecule was injected. For the binding studies, the column was equilibrated with the binding buffer for 5 CV. After sample injection, the injection loop was emptied with 1 CV and the column was washed with 7 CV of binding buffer. Once washed, elution step with 1 M Tris-HCl, pH 8.5 was applied, using a step or a linear gradient. For the step gradient elution studies, the previous protocol was applied. 50 mM phosphate was the binding buffer and different concentrations of Tris, sorbitol and arginine at pH 8.5 were used as elution buffers for 5 CV. After elution, the column was stripped with 5 CV of 1 M Tris pH 8.5 to remove any molecules still attached to the ligand, and regenerated with 5 CV of 1 M NaOH. In the case of gradient elution studies, the only difference was the application of a 10 CV gradient elution step and hold for 5 CV at 100% of elution buffer.

Characterization of target molecules

The isoelectric point (pI) of labelled and non-labelled Gammanorm®, proteins from CHO-S supernatant, Fc and Fabs fragments was determined by performing an IEF, following the protocol used in Pinto et al., 2015[12]. In addition, molecular weight of antibody-derived fragments was determined by SDS-PAGE, using the protocol described by the same authors.

Results and discussion

Screening and Optimization of Gammanorm® digestion

The first objective of this work was to screen and optimize the digestion reaction of Gammanorm® polyclonal antibody mixture. To narrow down the number of studied factors in further experiments, a first screening of several variables thought to affect IgG digestion was carried out, by changing one factor at a time. This included reaction time, reaction volume, IgG and papain concentrations, temperature and pH, in a total of 6 different parameters. After enzymatic cleavage, digestion efficiency was calculated by the amount of undigested immunoglobulin present in the digestion mixture, quantified using an analytical protein A column. At time zero, antibody molecules are intact, corresponding to the maximum absorbance elution area. On the other hand, after digestion, only Fc fragments will bind to the column. Since each antibody has one Fc and two Fab fragments, with similar molecular weights, if all immunoglobulin molecules are digested (100% digestion), then a 67% reduction in the elution peak area is expected. The results can be observed in **Figure 1.** The first digestion parameters studied were digestion time and papain concentration (**Figure 1 A**). In three of four concentrations tested, nearly 50% of the immunoglobulin molecules were digested during the first two hours and, after that, a less significant increase in antibody cleavage is observed over time. Results were similar between all papain concentrations, except 0.01 mg/mL, which was significantly less efficient, compared with the other concentrations used. Then, different reaction volumes and IgG concentrations were tested (**Figure 1 B**). In this case, higher digestion yields were obtained for lower IgG concentrations, possibly caused by enzyme saturation, while no significant differences could be seen for different reaction volumes. No clear correlation could be stablished when testing different buffer types and pH values (**Figure 1 C**). Finally, the impact of temperature on IgG cleavage was also tested, which showed that higher temperatures lead to higher digestion yields (**Figure 1 D**). Since the effect of some of the factors was not clear, MODDE software was used to treat the data, which allowed the development of a model describing IgG digestion in function of these variables. The impact of each factor on IgG cleavage was further analysed and led to the exclusion of reaction volumes and pH, once they were not being relevant to define final response.

Based on the previous results, a DOE approach was used to conduct the experiments, which allows the reduction of the number of experiments needed to extract a large set of data, by changing two factors at a time. Therefore, a total number of 18 experiments were designed using MODDE software. After performing these trials, data was processed by the same software and used to develop another model describing antibody cleavage in function of digestion time, substrate and enzyme concentrations and temperature. The model obtained presents a R^2 and Q^2 values of 0.92 a 0.82, respectively, corresponding to a robust model. The impact of each factor on digestion yield was further assessed and showed similar results to those obtained before, but in this case, papain concentration was the most important factor positively influencing the response, followed by temperature and digestion time. Moreover, substrate concentration has a negative impact on it, but less relevant than initial foreseen from previous results. Lastly, the optimizer tool was used to predict the optimum conditions for antibody enzymatic cleavage, which theoretically would lead to 92% digestion, when using 40ºC, 24 hours of digestion, 0.2 mg papain/mL and 1 mg IgG/mL. When testing these conditions, 95% cleavage was achieved, representing a 41% increase, compared to the initial conditions.

Overall, the presented DOE approach allowed the extraction of a large set of information from a minimal number of experiments, saving resources in the process. As kinetics and yields of enzymatic digestion vary according to the immunoglobulin subclass being used, this protocol should be tested and adapted when working with other types of antibodies. However, since the antibody mixture used for this optimization studied - Gammanorm® - has a polyclonal nature, the protocol herein described could be seen as a suitable initial step in the implementation of a papain-based digestion protocol.

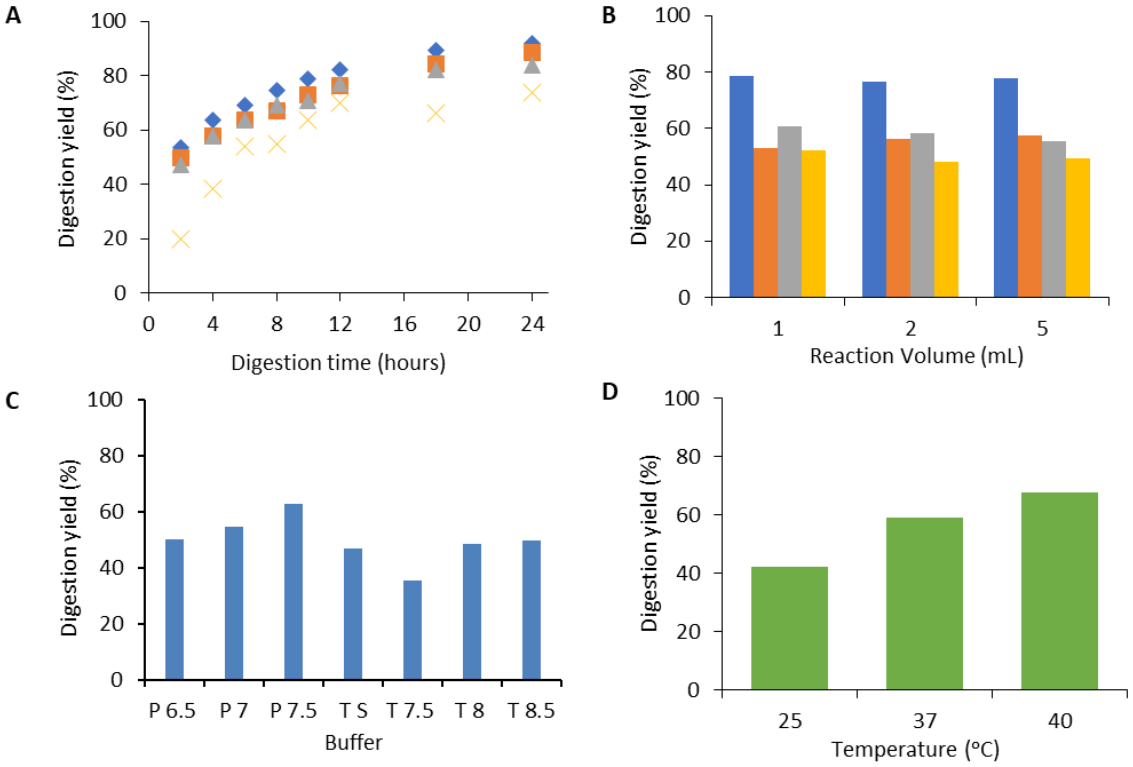

Figure 1. Impact of several factors on IgG digestion (%): **(A).** Digestion time (hours) and papain concentration (mg/mL). Blue: 0.2 mg papain/mL; Orange: 0.1 mg papain/mL; grey: 0.05 mg papain/mL; yellow: 0.01 mg papain/mL; **(B)**. Reaction volume (mL) and IgG concentration (mg/mL). Blue: 1 mg IgG/mL; Orange: 2 mg IgG/mL; Grey: 5 mg IgG/mL; Yellow: 10 mg IgG/mL **(C)**. Buffer type and pH values. P 6.5, 7 and 7.5: 10 mM phosphate buffer pH 6.5, 7 and 7.5, respectively; T S: 10 mM Tris buffer 100 mM NaCl pH 8; T 7.5, 8 and 8.5: 10 mM Tris buffer pH 7.5, 8 and 8.5, respectively. **(D)**. Temperature (ºC). The following standard conditions were used, except when indicated: 0.1 mg papain/mL, 6 hours of digestion, 1 mg IgG/mL, 1 mL total volume, 0.01 M phosphate buffer pH 7.4, 37ºC.

Downstream processing and characterization of Fab fragments

To obtain isolated labelled fragments for the following experiments, based on a microfluidic device, the Gammanorm® antibody mixture was labelled with BODIPY™ TMR NHS, digested using the previously optimized protocol and, finally, summited to a downstream protocol. This step is comprised of two consecutive chromatographic affinity steps – Protein A and Protein L - and led to the fractionation of lambda Fabs, kappa Fabs and Fc fragments. Flow-through and elution samples from both affinity processes, were further analysed by SDS-PAGE (**Figure 2**). Since protein A has affinity to the Fc fragment, the elution fraction (lane 4) contains different whole IgG molecules - different isotypes (two bands in the top part of the gel), Fc fragments (a band around 50 kDa) and other small digested fragments, which retained its affinity to the protein A ligand (a band around 25 kDa). The flow-through fraction (lane 3), containing kappa and lambda Fabs and other components from the digestion mixture, is injected to a protein L-affinity chromatography. This ligand has affinity to Fabs containing a k light chains, but not to those containing lambda light chain. As a consequence, in the elution (lane 6) kappa Fabs from different isotypes of IgG (a band around 50 kDa) and other digested fragments with affinity to protein L are present (a band below 25 kDa), for example, free light chains. In Protein L flow-through (sample 5), lambda Fabs (a fade band around 50 kDa), other digested fragments without affinity to neither protein A or protein L, along with compounds from the digestion mixture, including papain (around 23 kDa), can be found.

For the high throughput screening of resins and chromatographic conditions, some methods are based on fluorescent measurements of fluorophores coupled to target molecules. However, the attachment of these fluorescent molecules can, in many cases, affect the properties of the target molecules, thus influencing the results[13].

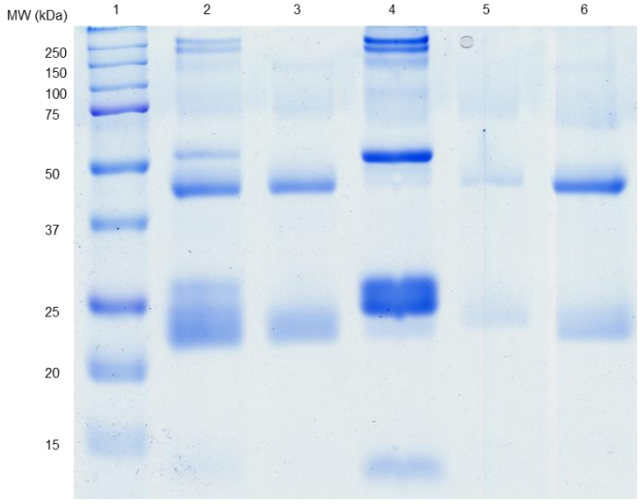

Figure 2. SDS-PAGE gel of all the fractions collected in the downstream processing of polyclonal IgG digestion mixture. **1** - Molecular weight ladder; **2** - Digestion Mixture; **3** - protein A flow-through; **4** - protein A elution; **5** - Protein L flow-through; **6** - protein L elution. Digestion were carried out using the following conditions: 24 hours of digestion, 0.20 mg papain/mL, 1 mg IgG/mL, 40ºC.

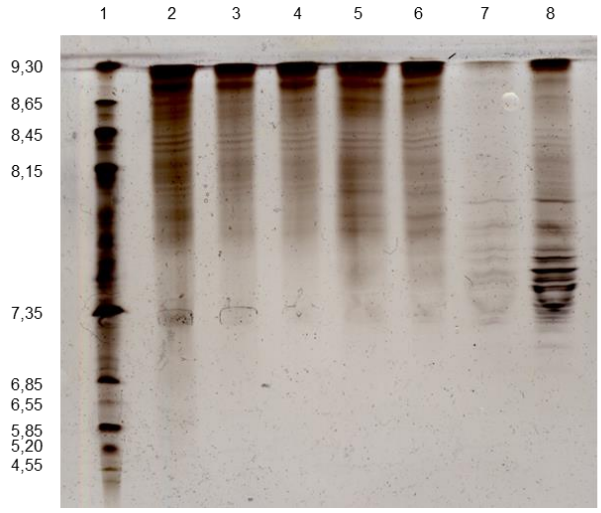

Figure 3. Isoelectric focusing gel of Gammanorm® derived fragments **1** - Marker; **2**- Labelled kappa Fabs (BDP FL NHS); **3** - Non-labelled kappa Fabs; **4** - Labelled kappa Fabs (BDP TMR NHS); **5** - Non-labelled lambda Fabs; **6** - Labelled lambda Fabs (BDP TMR NHS) ; **7** - Non-labelled Fc fragments; **8** - Labelled Fc fragments (BDP TMR NHS).

Because of that, the IEF technique was applied to check if the coupling of BODIPY™ TMR NHS to IgG fragments was affecting their properties, allowing the determination of pI ranges from Fc and kappa and lambda Fab fragments and the comparison between labelled and non-labelled proteins (Figure 3). Also, kappa Fab fragments labelled with another fluorophore, BODIPY™ FL NHS ester, were analysed. In all the cases, the multiple bands observed for labelled proteins were also visible in the corresponding non-labelled samples, meaning they have similar charges. In addition, the IEF gel presented several bands in each lane, which can be explained by the fact that Gammanorm® IgG is a mixture of antibodies, and so, different isotypes and subclasses with varying isoelectric points are present in solution.

Overall, these results suggest that both fluorophores analysed can be used as a tool for downstream studies employing high throughput methods based on fluorescence, specially purification methods based or affected by charge phenomena. Nonetheless, the hydrophobicity conferred by the phenyl groups of BODIPY fluorophores must be taken into account, since they can enhance hydrophobic interactions, leading to less accurate results[13].

High-throughput screening of chromatographic conditions using phenylboronate as a multimodal ligand

Binding Studies

To assess the possibility of using aminophenylboronate as a multimodal ligand with affinity for cis-diol groups, present in kappa and lambda Fabs, a microfluidic platform developed by Pinto and co-workers was used, which allows the rapid screening of operational conditions with a minimum consumption of reagents and biomaterial[8]. After performing the experiments, fluorescence data was extracted and adjusted to the Hill model, allowing the determination of the F_{max} for each condition. This parameter corresponds to the maximum fluorescence intensity value when ligand is saturated. The device was firstly employed to test different adsorption conditions, including 5 different salt concentrations, ranging from 0 mM to 200 mM of NaCl, and 3 pH values, including 5, 7 and 9.2, in 50 mM acetate, phosphate and carbonate buffers, respectively. Also, 1 M of NaCl was tested to confirm that a sharp increase in salt concentration would not considerably affect adsorption, which was true for all cases. The results represented in Figure 4. were normalized considering the highest Fmax for each target molecule: 24.9 a.u. at pH 5 using 150 mM NaCl and 27.6 a.u. at pH 5 using 50 mM NaCl, for kappa (DOL=0.344) and lambda (DOL=0.301) respectively. Looking at the presented heatmaps, binding of kappa and lambda Fabs to aminophenylboronate was enhanced at pH 5, under intermediate ionic strength, and at pH 7, using no salt or lower salt concentrations. In contrast, at pH 9.2 and for all salt concentrations tested, Fmax values are lower, which means a less significant binding is taking place under these conditions. In fact, when environmental pH is higher than boronic acid pKa, around 8.8-8.9, aminophenylboronate adopts a tetrahedral conformation, which is negatively charged[14]. Because the pI of most of Fabs is lower than 9.2, as it was previously observed in Figure 3, negatively charged fragments are predominant, resulting in electrostatic repulsion between ligand and target molecules. This phenomenon is probably leading to suppression of specific binding, since Fabs are not able to make the necessary surface contact with the ligand. Interestingly, an increase in ionic strength should have been enough to supress electrostatic interactions and enhance affinity binding, but this was not observed, even when using 1 M NaCl[15]. Regarding the results at pH 5 and 7, aminophenylboronate is expected to be predominantly in a neutral trigonal conformation, which has lower affinity towards molecules containing cis-diol groups. Yet, in this conformation, the ligand has an empty orbital in the boron atom, becoming a Lewis acid ready to accept a pair of electrons from a Lewis base. Potential electrons donors include unprotonated amines or carboxyl groups present in several amino acids or even components of the adsorption buffers, such as phosphate (PO_4^{-3}), acetate ($C_2H_3O_2^-$) or the chloride ion (Cl^-) from NaCl. Either way, when this coordination reaction happens, ligand conformation is switched to tetrahedral, promoting in some cases higher affinity interactions and PB specificity[16,17]. Thus, since these Lewis bases are present in all the conditions tested, this secondary interaction is probably taking place, enhancing primary interaction of Fabs to aminophenylboronate. Moreover, at these pH values, fragments tend to be positively charged, since none of them present an isoelectric point below 7.35 (see Figure 3). This means electrostatic attraction between negative ligand and positive Fabs can be occurring, increasing protein retention. None the less, even if this is happening, electrostatic interactions seem not to be the driving force of the chromatography process, since the use of higher salt concentrations did not have a significant impact on the results. Other possibility is that hydrophobic interactions, which are enhanced under a higher ionic strength, can be balancing the suppression of the electrostatic interactions. Then, for the same operating conditions, other target molecules were further assessed, including impurities from of enzymatic cleavage of antibody, namely both full-sized antibodies (DOL=2.75) and Fc fragments (DOL=0.277), and from recombinant production using mammalian cells namely molecules present in a CHO-S supernatant, including undesirable proteins, lipids and nucleic acids produced by the host cells. The results obtained are shown in Figure 5. In all the cases, binding to aminophenylboronate was less significant at pH 9.2, probably because of the electrostatic repulsion discussed above. Moreover, while undigested antibody (maximum value of Fmax = 23.9 u.a.) and CHO-S supernatant adsorption (maximum value of Fmax = 76.8 u.a.) did not significantly changed with ionic strength, binding of Fc to

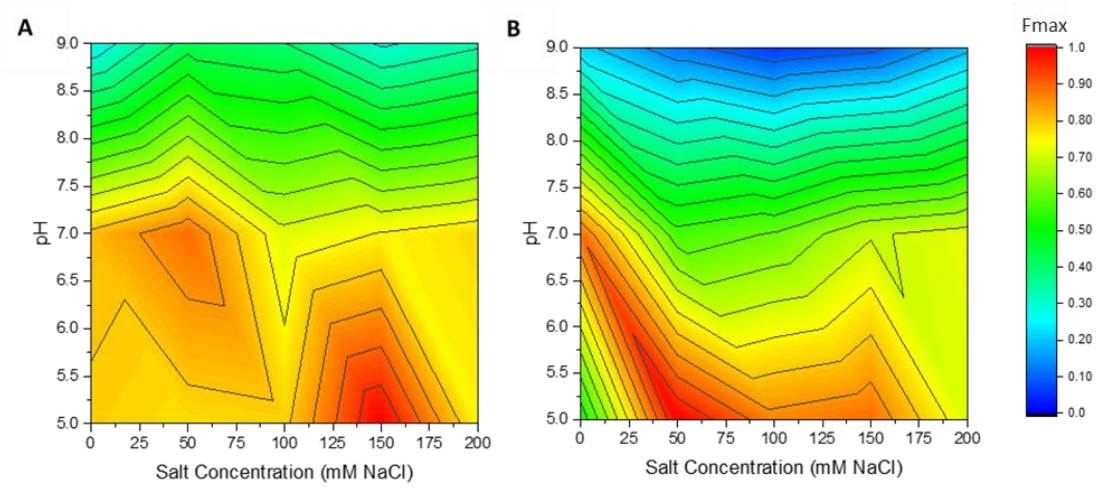

Figure 4. Relative adsorption of kappa (**A**) and lambda (**B**) Fab fragments to aminophenylboronate, under different pH values (5,7 and 9.2) and salt concentrations (ranging from 0 to 200 mM NaCl). Fluorescence values were adjusted to the Hill enzymatic kinetics model, which produced a Fmax for each condition, corresponding to maximum adsorption when ligand is saturated. Results were normalized, considering the highest value. DOL kappa Fabs: 0.344; DOL lambda Fabs: 0.301.

aminophenylboronate was maximized using 200 mM NaCl at pH 7 and no added salt at pH 5, presenting Fmax of 13.6 and 11.9 u.a., respectively. This can suggest the presence of hydrophobic and electrostatic interactions, which are enhanced in the presence of higher and lower ionic strengths, respectively[15]. Since, below ligand pKa, adsorption of Fab and Fc fragments is salt-dependent, on contrary to what was observed for the whole antibody, ionic and hydrophobic interactions are probably having a more relevant role in first case. In fragments, the digestion of the antibody can result in the exposure of hydrophobic and charged amino acids, being those proteins more prone to the establishment of hydrophobic/electrostatic interactions.

Elution Studies

The next step was to screen different elution conditions of Fabs from aminophenylboronate at microscale. The method used was adapted by adding an additional step, which consisted in flowing the elution buffer into the microchannel under a fluorescence microscope. This allowed the monitoring of fluorescence decay as Fabs were being eluted from the column. A total of six different elution agents were tested, including Tris, D-sorbitol and magnesium chloride at concentrations ranging from 0 to 2 M and guanidine hydrochloride, urea and arginine at lower concentrations from 0 to 1 M. Also, for all of them the pH was maintained at 8.5, by adding 150 mM Tris-HCl. Adsorption was performed using 50 mM phosphate buffer at pH 7, with no salt added, which was also used as a negative control during elution. Results for all the conditions tested can be observed in **Figure 6**. Slope of the decrease of fluorescence intensity with time for each condition were calculated for the first 15 seconds. Looking at the results, elution of Fabs from aminophenylboronate was more efficient using Tris as elution buffer. For this agent, the best conditions were 0.5 M Tris-HCl for kappa (-3.36 %/s) and 1.75 M Tris-HCl for lambda Fabs (-4.32 %/s). In fact, Tris has been used to remove full-size monoclonal antibodies from phenylboronate, due to its capacity to disrupt not only affinity bonding, but also secondary interactions, working as a competitor for the ligand via esterification and coordination interaction[6]. The best results obtained with Tris were followed by guanidine hydrochloride and arginine, also effective in eluting Fabs, being the best conditions 1.0 M guanidine hydrochloride (-2.96 %/s) and 0.5 M arginine (-2.87 %/s) in the case of kappa, and 0.5 M arginine (-4.01 %/s) and 1.0 M guanidine hydrochloride (-3.70 %/s), in the case of lambda Fabs. Besides supressing aggregation and promoting protein renaturation, arginine has been used as elution agent for monoclonal antibodies in several types of chromatography, including protein A affinity chromatography and multimodal cation exchange chromatography (e.g. Capto MMC, which is based on hydrophobic and electrostatic interactions). It affects all the secondary interactions present in aminophenylboronate multimodal chromatography, such as hydrophobic and electrostatic interactions and hydrogen bonding, as well as affinity interactions, because of its tendency to accumulate in protein surfaces, specifically near aromatic, polar and charged aminoacids, reducing interaction with the column[18,19]. On the other hand, guanidine, as well as urea, are denaturing and chaotropic agents, responsible for disruption of hydrophobic interactions and other non- electrostatic interactions[18]. In the case of denaturing agents, change in tertiary structure can lead to loss of affinity to the ligand. Therefore, the most efficient agents were the ones interfering with both affinity and non-affinity binding. Producing worst elution results were D-sorbitol (maximum slopes of -2.01 %/s and -3.00 %/s, for 1.0 M and 1.5 M sorbitol and kappa and lambda Fabs, respectively), a competitor to specific interactions with high affinity towards APB[20], magnesium chloride (maximum slopes of -2.62 %/s and -1.31 %/s, for 0.25 M $MgCl_2$ and kappa and lambda Fabs, respectively), a chaotropic salt which affects electrostatic interactions and possibly π-π complexation but has been reported as an enhancer of cis-diol affinity interactions[5], and urea (maximum slopes of -1.67 %/s and 2.75 %/s, for 0.75 M and 0.5 M urea and kappa and lambda Fabs, respectively).

Overall, results show that agents with the ability of interfering with both cis-diol affinity bonding, but also with non-covalent bonding, especially hydrophobic and electrostatic interactions,

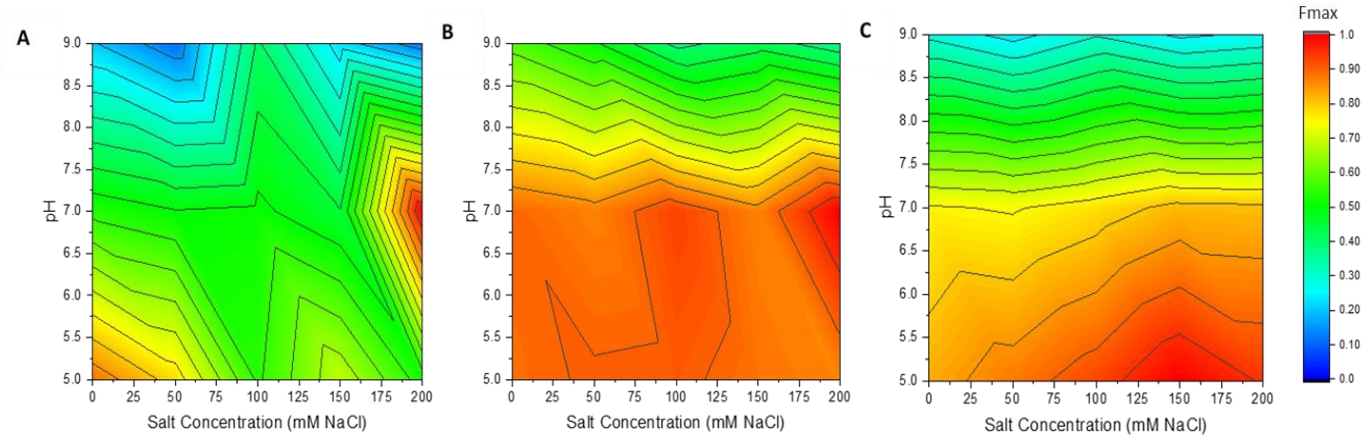

Figure 5. Relative adsorption of Fc fragments (**A**), undigested polyclonal antibody mixture (**B**) and proteins from CHO-S supernatant (**C**) to aminophenylboronate, under different pH values (5,7 and 9.2) and salt concentrations (ranging from 0 to 200 mM NaCl). Fluorescence values were adjusted to Hill model, which produced a Fmax for each condition, corresponding to maximum adsorption when ligand is saturated. Results were normalized, considering the highest value. DOL FC fragments = 0.277; DOL Gammanorm® = 2.75

generate maximized elution of Fabs from aminophenylboronate. On the other hand, D-sorbitol and magnesium, chloride, that only negatively affect primary or secondary interactions, respectively, are among the worst elution agents tested. This suggests that a synergetic effect between covalent affinity binding and secondary interactions is leading to target molecule adsorption.

Another relevant aspect is that, generally, maximum slopes were obtained for intermediate concentrations of elution agents, all equal to 1.0 M or lower, except for lambda Fabs when using Tris and D-sorbitol, which required the use of 1.75 and 1.5 M of elution agent, respectively, for maximized elution. This is clearly visible in the graphs, as results tend to become homogenous generally for concentrations higher than 0.5 M of elution buffer. In the case of magnesium chloride, the best results were achieved when using only 0.25 M $MgCl_2$, which can be explained by the fact that affinity binding is being enhanced, even though non-specific interactions are being supressed. The use of lower concentrations of the elution agent allows saving reagents, especially important for chromatographic processes using expensive reagents as arginine. In this case, maximum recover of target molecules was achieved using 0.5 M arginine for both classes of Fabs, half of the highest concentration tested.

Also important is the fact that, sometimes, divergent results were obtained for kappa and lambda Fabs. For example, while magnesium chloride could be used to elute the kappa isotypes, as a maximum slope of -2.62 %/s was achieved when using 0.25 M $MgCl_2$, in the second case slopes are not far from the negative control. The opposite situation was observed for urea. Moreover, the highest slope values were obtained for lambda isotypes, except in the case of magnesium chloride. This may indicate that different types of interactions are having varying relevance in the adsorption process of each class of fragments to aminphenylboronate and so, light chain plays an importance role in the process. The availability of different interacting groups may also be different in different isotypes of Fabs.

Concluding, the microfluidic approach used was useful to test a large set of operational conditions and target molecules in a relatively short period of time and in a cost-effective manner. However, in the case of adsorption, it does not allow direct comparison of the results between different target molecules, because they present a different number of fluorophores attached to their molecular structure, which can also be affecting the results. Moreover, it does not give any information about the proportion of molecules attached to the beads versus the molecules in the flow-through. So, studies at the macroscale are required to extract this information, but also for validation of the results at microscale.

Validation of the microfluidic results at macroscale

Adsorption studies

The next step was to validate the previous results at macroscale, using an ÄKTA™ Purifier 10 System, which allows protein detection by reading its absorbance at 280 nm. For this, a total of five conditions were chosen as representative of the data set produced at microscale: 50 mM phosphate buffer pH 7 for three different salt concentrations, including 0, 50 and 150 mM NaCl, 50 mM acetate buffer 150 mM NaCl pH 5 and 50 mM carbonate buffer 150 mM NaCl pH 9.2. 1 M Tris-HCl was chosen as the standard elution buffer, as it produced good results for Fabs elution. These conditions were tested for adsorption of both Fabs isotypes, as well as impurities from distinct fragments manufacturing method: Fc fragments (digestion) and CHO-S supernatant (recombinant production). A negative control consisted in the injection of adsorption buffer, whose chromatogram was subtracted to every chromatogram obtained. Also, considering the peak area corresponding to flow-through and elution, a ratio between elution and total area was calculated for each condition. In addition, a single injection of Gammanorm® IgG was performed at pH 7 with no salt added, which resulted in the recovery of 99.24% of proteins in the elution fraction. Finally, SDS-PAGE analysis for different fractions corresponding to kappa and lambda Fabs FT and E was performed with silver staining for better visualization of the protein bands. Gels can be observed in **Figure 7** and **8**. The results obtained at macroscale follow the conclusions of the studies at microscale. Firstly, at pH 9.2, adsorption of fragments is less significant than at pH 5 and 7, as the FT fraction shows higher peak area and the E fraction is almost inexistent. As mentioned before, under these conditions low binding occurs probably due to electrostatic repulsion, which does not allow the protein to make the surface contact needed

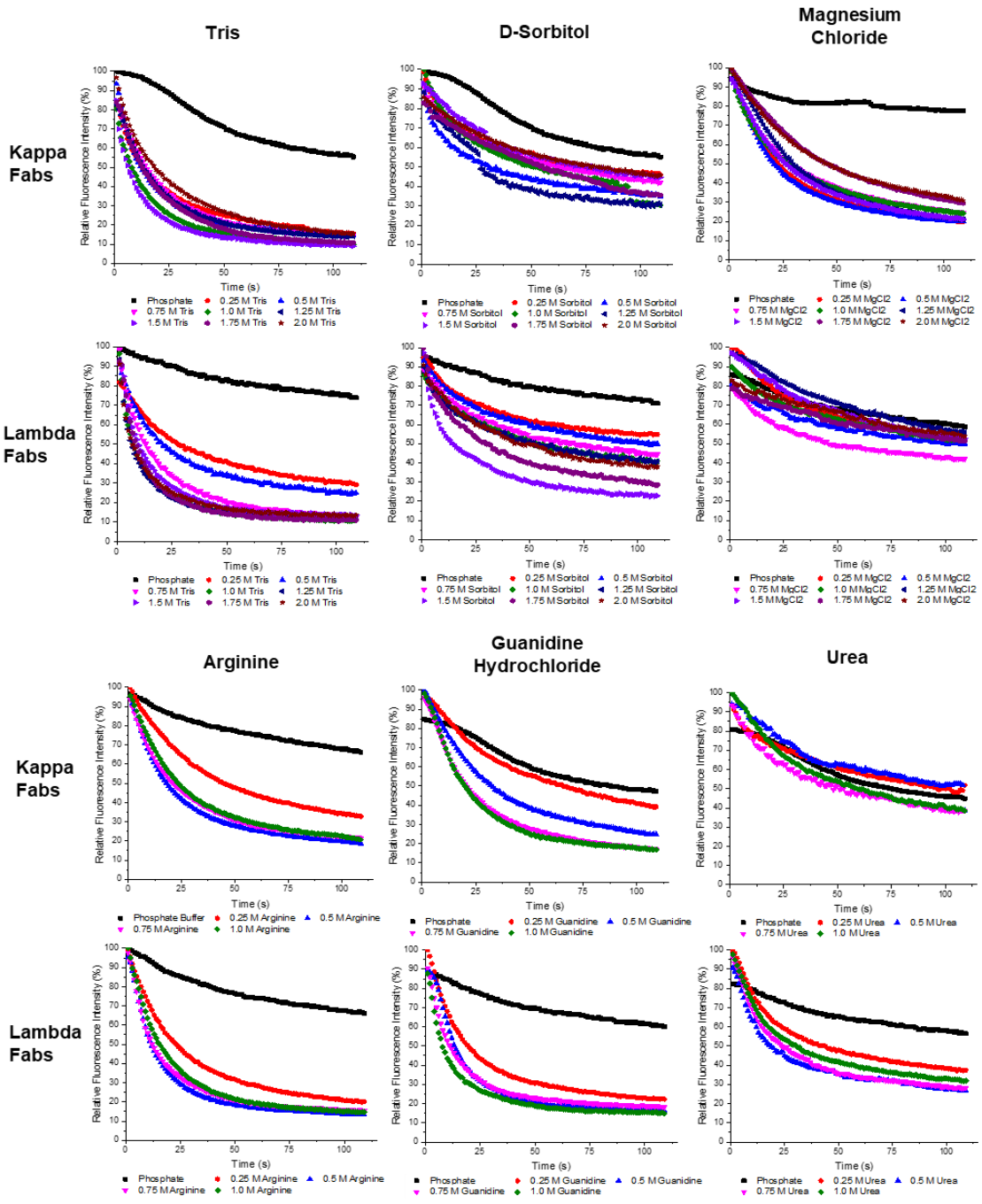

Figure *6*. Decay of relative fluorescence over time, corresponding to elution of kappa and lambda Fabs from aminophenylboronate, using different elution buffers: Tris, Sorbitol, Magnesium chloride, Arginine, Guanidine hydrochloride and Urea. All of them contain 150 mM Tris-HCl as buffer to maintain a pH of 8.5. Adsorption is performed using 50 mM phosphate as binding buffer.

for the establishment of the covalent bonds. In opposition, binding at pH 5 and 7 is much higher for antibody-derived fragments, as a maximum increase of 14.68, 26.73 and 73.38 % in ratios of E/total peak areas were observed for kappa and lambda Fabs and Fc fragments, respectively, when comparing with results obtained at pH 9.2. Under these conditions, coordination interaction is probably responsible for switching the ligand from the trigonal to the tetrahedral conformation, promoting the affinity covalent interactions. Moreover, electrostatic attraction between positive target molecules and negative ligand can be involved. In the case of proteins from CHO-S supernatant, results were approximately the same at pH 5, 7 and 9.2, because these impurities have almost no binding for all tested conditions. In the microfluidic studies, these components were observed to have a significantly higher binding at pH 5 and 7 than at pH 9.2, but this did not happen here, probably because other labelled components of the supernatant, not read at 280 nm, are interfering with the results at microscale. For all target molecules,

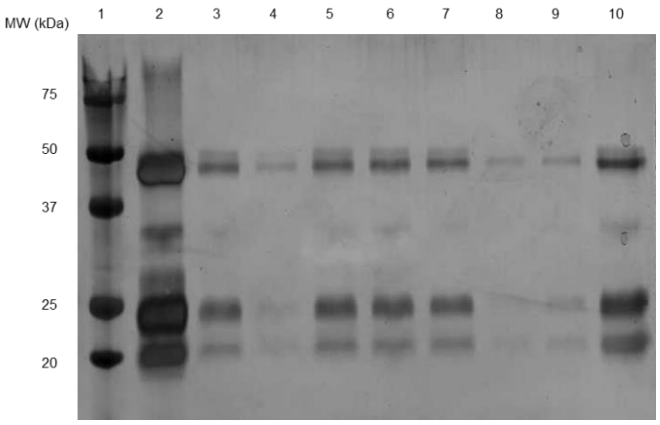

Figure 7. Silver stained SDS-PAGE gel for different fractions of aminophenylboronate chromatography, using kappa Fabs as target molecules. 50 mM Phosphate pH 7 and 1 M Tris-HCl pH 8.5 were used as adsorption and elution buffers, respectively. **1** - Protein Marker; **2** - Injection Sample; **3** - Pool of FT fractions; **4** to **6** - FT fractions; **7** - Pool of E fractions; **8** to **10** - E fractions

the ratio between elution and total area was similar for different NaCl concentrations at pH 5 and 7, except for kappa Fabs and Fc fragments, which demonstrate lower binding when using acetate and phosphate 150 mM NaCl, respectively, as binding buffer. These results suggest the importance of electrostatic interactions in those cases, as a suppression of these interactions is observed when ionic strength is increased.

Apart from the validation of the previous results, experiments at macroscale also allowed comparative results between different biomaterials and the measurement of protein that binds to the column versus the protein in the FT, not possible at microscale. For example, CHO-S supernatant was almost completely recovered in the FT fraction, proving its inability to bind to aminophenylboronate under all the tested conditions, even though it showed the highest fluorescence values in the screening step. This was probably due to the fact that not only proteins were labelled with the fluorophore for the microfluidic experiments, but also other molecules present in the cell culture supernatant (lipids, nucleic acids and other compounds), that are not detected at 280 nm. In opposition, Gammanorm® IgG had almost 100% of recovery in the E fraction, while the fragments showed a different binding to the ligand. Fc fragments had a maximum recovery of 85.40%, while lambda and kappa Fabs had a value of 48.35% and 34.80%, respectively. The results obtained suggest one of two things: Fc and Fab regions have a combined effect on binding of the whole antibody to APB or the hinge region, where papain cut the IgG molecule, is important in the adsorption process of full-sized antibodies. In addition, enzymatic cleavage can be affecting tertiary structure, decreasing fragments retention in the column, when compared with the respective antibody.

Moreover, looking at SDS-PAGE gels, it can be observed that bands around 50 kDa, corresponding to Fabs, and other bands around 25 kDa, corresponding to smaller fragments, such as free light chains, are present in both the FT and E fractions. Since the experiments were carried out using fragments obtained from the enzymatic cleavage of polyclonal antibodies, it is possible that differential separations are occurring according to the IgG isotype. To prove this, further tests would be needed. If this is true, aminophenylboronate could be used as a tool to purify certain isotypes of Fabs, but not as a universal tool as it is observed for Gammanorm®. Also, it would only be useful in two situations: for the downstream processing of fragments produced in recombinant systems, in the case where fragments totally bind to the column and proteins present in CHO-S supernatant are washed out, or for fragments produced by enzymatic cleavage if Fab fragments show no affinity towards the ligand and the remain impurities adsorbed to aminophenylboronate.

Elution studies

A validation of the elution results obtained with the microfluidic chromatographic device was also performed at macroscale, using the same procedure described for the adsorption studies. For this, Tris and arginine were chosen once they produced the best results, along with D-sorbitol, which was the only elution agent used that interferes solely with cis-diol affinity interactions. A concentration of 150 mM Tris-HCl pH 8.5 was tested as a control. Although guanidine hydrochloride produced good results in the microfluidic studies, it was excluded because of its protein denaturating activity. For the three elution agents, a linear gradient elution and a step gradient elution of Fabs at three different buffer concentrations, including 20, 35 and 50% of elution buffer**,** were performed. Here, apart from the confirmation of the results for kappa and lambda Fabs, also Fc fragments and Gammanorm® IgG elution were studied, to check the possibility of differential retention in the column. A negative control consisting of adsorption buffer was carried out and subtracted to each chromatogram obtained. After the chromatographic runs, the efficiency of each buffer was assessed by calculating the ratios of elution peak to total area. In the case of whole antibody, protein was totally recovered in the FT fraction.

Tris and sorbitol led to the acquisition of the best results, followed by arginine. Using Tris as an elution agent, Gammanorm® IgG was entirely collected in the E fraction, with maximum peak height of around 480 mAU followed by Fc fragments, where around two thirds of the molecules were collected in the E fraction, showing peak heights of approximately 180 mAU. Fab fragments presented a lower recovery, with ratios E to total peak areas of about 30 and 40%, and peaks heights of 60 and 100 mAU, for kappa and lambda Fabs respectively. For D-

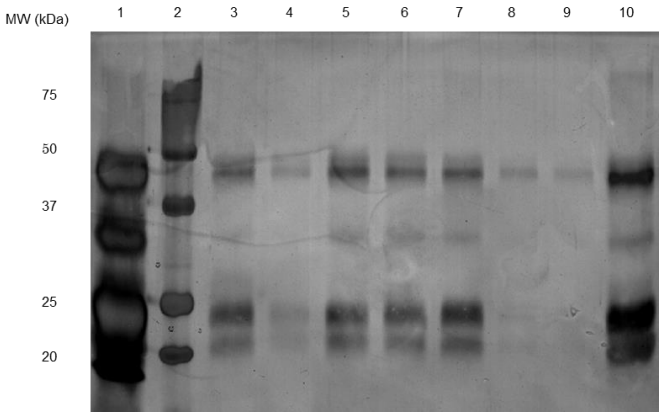

Figure 8. Silver stained SDS-PAGE gel for different fractions of aminophenylboronate chromatography, using lambda Fabs as target molecules. 50 mM Phosphate pH 7 and 1 M Tris-HCl pH 8.5 were used as adsorption and elution buffers, respectively. **1** - Injection Sample; **2** - Protein Marker; **3** - Pool of FT fractions; **4** to **6** - FT fractions; **7** - Pool of E fractions; **8** to **10** - E fractions.

sorbitol, a second smaller peak is observed when stripping the column with 1 M Tris-HCl, meaning the competing agent is not able to elute all the proteins attached to the column. A control elution, using 150 mM Tris-HCl, which is present in every elution buffer, resulted in the elution of only a small fraction of the proteins and so, its addition to D-sorbitol and arginine buffers is not having a key role in the elution process.

In the step gradient elution runs, the higher the concentration of elution buffer, the closer the results would get from those obtained in the linear gradient runs and, for 50% of buffer, the results obtained in the two elution gradients were approximately the same. Here, the presence of a second elution peak is clear, when flowing 1 M Tris-HCl into the column, for all tested step concentrations of Tris, in the case of kappa Fabs, and for all the step concentrations tested of D-sorbitol and for lower concentrations of Tris and arginine in the case of lambda Fabs.

In contrast to what was concluded from the results at microscale, affinity cis-diol interactions seem to be the driving force for retention of antibodies and fragments in the column, since sorbitol, affecting only cis-diol primary binding, and Tris, affecting both affinity and non-affinity interactions, showed similar results. For the same reason, it can be concluded that secondary interactions do not having an important role in the process, except for lambda Fabs, as a second elution peak is observed when stripping the column with 1 M Tris-HCl, after testing sorbitol buffer. In the remaining cases, given the small impact of these types of chemical bonding (e.g. electrostatic and hydrophobic interactions) on protein retention, the presence of 150 mM Tris-HCl in the sorbitol buffer may be enough to supress them. Also, since elution of all target molecules occurred at the same time, separation of Fabs from the Fc fragments and Gammanorm® IgG by differential elution is not possible.

Overall, the microfluidic device used to screen chromatographic conditions was useful and predicted best binding and elution conditions for each target molecule. However, when using D-sorbitol as the elution agent, results at macroscale did not reflected the patterns observed at microscale, where Tris produced the best results, followed by arginine. This may be caused by the utilization of different chromatographic conditions, particularly different superficial velocities, as buffers enters the microcolumn (210 nL) at 15 µL/min, while in the ÄKTA™ Purifier 10 System, buffers flow into the 1 mL column at 1 mL/min. Because of this, in the first case, residence time of D-sorbitol is 0.28 s, too short for the agent to compete with the target molecules for the binding to aminophenylboronate, while in the case is 60 s. Thus, a higher residence time would be required to mimic with more precision conditions generally used at lab scale.

Conclusions and Outlook

In the current work, the possibility of using aminophenylboronate as an affinity ligand in the downstream processing of antibody fragments was assessed, using a microfluidic device previous developed by Pinto and coworkers, which is based on fluorescence measurement of labelled proteins. Prior to this, screening and optimization of factors involved in the manufacturing process of Fab fragments, by enzymatic cleavage of a polyclonal mixture of antibodies, were performed. Under the optimum values of digestion time, temperature and papain and antibody concentrations, an increase of 41% in antibody digestion yield was achieved. Screening of binding conditions for kappa and lambda Fabs and impurities tested showed that binding at pH 9.2 is less significant for all of them, probably due to electrostatic repulsion between negatively charged proteins and ligand. At pH 5 and 7, proteins could bind to the column, as cis-diol affinity binding seems to be the prevalent force, enhanced by coordination reaction between a Lewis base and the ligand. Maximum binding of Fab fragments was achieved when using intermediate salt concentrations at pH 5 and lower ionic strength at pH 7. Then, elution studies at microscale showed more elution of Fabs from the column using agents affecting both specific and secondary interactions, which was the case of tris, arginine and guanidine hydrochloride. When validating these results at macroscale using an ÄKTA™ Purifier 10 System, more data could be extracted, including the ratio of protein in the elution fraction to the total of protein injected and comparison of results between different target molecules. During validation of the adsorption results, global trends observed at microscale could be confirmed. Moreover, proteins from CHO-S supernatant were almost totally recovered in the FT fraction, while the opposite situation happened for undigested Gammanorm® IgG. Fc and Fab fragments where present in both FT and E fractions. Finally, when validating elution results, tris and D-sorbitol produced similar protein elution from the column, suggesting that cis-diol affinity bonding, enhanced by coordination secondary interaction, is in fact the driving force for retention of the target molecules tested.

Given the results obtained for fragments derived from polyclonal antibody mixture, aminophenylboronate could still be used in the downstream processing of kappa and lambda Fabs, but only in two situations: the fragments are expressed in CHO-S cells and will totally binds to the ligand while impurity proteins from the cell culture supernatant are wash-out or the fragment is produced by enzymatic cleavage and it remains in the FT fraction while Fc fragments and undigested antibody bind to the column. Either way, further studies would be required to confirm these possibilities. Also, it would be important to confirm if the chromatographic conditions here used do not significantly change protein tertiary structure and its bioactivity

Acknowledgments

This work was funded by the European Union 7th framework program under grant agreement no.312004. iBB acknowledges Fundação para a Ciência e Tecnologia (FCT) for funding through the research unit (FCT ref. UID/BIO/04565/2013) and project PureMab (FCT Ref. PTDC/QEQ-PRS/0286/2014).

References

1. Lipman, N. S., Jackson, L. R., Trudel, L. J. & Weis-Garcia, F. Monoclonal Versus Polyclonal Antibodies: Distinguishing Characteristics, Applications, and Information Resources. *ILAR J.* **46**, 258–268 (2005).
2. Steeland, S., Vandenbroucke, R. E. & Libert, C. Nanobodies as therapeutics: Big opportunities for small antibodies. *Drug Discov. Today* **21**, 1076–1113 (2016).
3. Rodrigo, G., Gruvegård, M. & Van Alstine, J. Antibody Fragments and Their Purification by Protein L Affinity Chromatography. *Antibodies* **4**, 259–277 (2015).
4. Spitali, M. Downstream processing of monocloanl antibody fragments. in *Process Scale Purification of Antibodies* (U. Gottschalk (Ed.), 2017).
5. Brena, B. M., Batista-Viera, F., Rydén, L. & Porath, J. Selective adsorption of immunoglobulins and glucosylated proteins on phenylboronate-agarose. *J. Chromatogr. A* **604**, 109–115 (1992).
6. Azevedo, A. M. *et al.* Capture of human monoclonal antibodies from a clarified cell culture supernatant by phenyl boronate chromatography. *J. Mol. Recognit.* **23**, 569–576 (2010).

7. Nascimento, A. *et al.* Studies on the purification of antibody fragments. *Sep. Purif. Technol.* **195**, 388–397 (2018).
8. Pinto, I. F. *et al.* High-Throughput Nanoliter-Scale Analysis and Optimization of Multimodal Chromatography for the Capture of Monoclonal Antibodies. *Anal. Chem.* **88**, 7959–7967 (2016).
9. São Pedro, M. N., Azevedo, A. M., Aires-Barros, M. R. & Soares, R. R. G. Minimizing the Influence of Fluorescent Tags on IgG Partition in PEG–Salt Aqueous Two-Phase Systems for Rapid Screening Applications. *Biotechnol. J.* **14**, 1800640 (2019).
10. Pinto, I. F. *et al.* The application of microbeads to microfluidic systems for enhanced detection and purification of biomolecules. *Methods* **116**, 112–124 (2017).
11. Andrew, SM., Titus, J. Fragmentation of Immunoglobulin G. in *Current Protocols in Cell Biology* **17**, 16.4.1-16.4.10 (2003).
12. Pinto, I. F., Rosa, S. A. S. L., Aires-barros, M. R. & Azevedo, A. M. Exploring the use of heparin as a first capture step in the purification of monoclonal antibodies from cell culture supernatants. *Biochem. Eng. J.* **104**, 27–33 (2015).
13. São Pedro, M. N., Azevedo, A. M., Aires-barros, M. R. & Soares, R. R. G. Minimizing the influence of fluorescent tags on IgG partition in PEG-salt aqueous two-phase systems for rapid screening applications. *Biotechnol. J.* **14**, (2019).
14. Li, D., Chen, Y. & Liu, Z. Boronate affinity materials for separation and molecular recognition: Structure, properties and applications. *Chem. Soc. Rev.* **44**, 8097–8123 (2015).
15. Carvalho, R. J., Woo, J., Aires-Barros, M. R., Cramer, S. M. & Azevedo, A. M. Phenylboronate chromatography selectively separates glycoproteins through the manipulation of electrostatic, charge transfer, and cis-diol interactions. *Biotechnol. J.* **9**, 1250–1258 (2014).
16. Rosa, S. A. S. L., da Silva, C. L., Aires-Barros, M. R., Dias-Cabral, A. C. & Azevedo, A. M. Thermodynamics of the adsorption of monoclonal antibodies in phenylboronate chromatography: Affinity versus multimodal interactions. *J. Chromatogr. A* **1569**, 118–127 (2018).
17. Rosa, S. A. S. L. *et al.* Mobile phase modulators as salt tolerance enhancers in phenylboronate chromatography: thermodynamic evaluation of the mechanisms underlying the adsorption of monoclonal antibodies. *Biotechnol. J.* 0–2 (2019). doi:10.1002/biot.201800586
18. Hirano, A., Shiraki, K. & Tomoshi, K. Effects of Arginine on Multimodal Chromatography: Experiments and Simutions. Current protein and peptide science. 20, 40-48 (2019).
19. Shukla, D., Zamolo, L., Cavallotti, C. & Trout, B. L. Understanding the Role of Arginine as an Eluent in Affinity Chromatography via Molecular Computations. *J. Phys. Chem.* **115**, 2645–2654 (2011).
20. dos Santos, R., Rosa, S. A. S. L., Aires-Barros, M. R., Tover, A. & Azevedo, A. M. Phenylboronic acid as a multi-modal ligand for the capture of monoclonal antibodies: Development and optimization of a washing step. *J. Chromatogr. A* **1355**, 115–124 (2014).