Journal of Chromatography B, 903 (2012) 163–170

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Fishing human monoclonal antibodies from a CHO cell supernatant with boronic acid magnetic particles

L. Borlido[a], A.M. Azevedo[a], A.G. Sousa[a], P.H. Oliveira[a], A.C.A. Roque[b], M.R. Aires-Barros[a,*]

[a] Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Department of Bioengineering, Instituto Superior Técnico, Technical University of Lisbon, Av. Rovisco Pais, 1049-001 Lisbon, Portugal
[b] REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

ARTICLE INFO

Article history:
Received 30 March 2012
Accepted 15 July 2012
Available online 20 July 2012

Keywords:
Magnetic particles
Boronic acid
Monoclonal antibody
Affinity purification
Downstream processing

ABSTRACT

In this work we have evaluated the potential of boronic acid functionalized magnetic particles for the one-step capture of a human monoclonal antibody (mAb) from a Chinese hamster ovary (CHO) cell culture supernatant. For comparison, Protein A coated magnetic particles were also used. The most important factor influencing the overall process yield and product purity in boronic acid particles was found to be the binding pH. Basic pH values promoted higher purities while resulting in decreased yields due to the competing effects of molecules such as glucose and lactate present in the cell culture supernatant. After optimization, the particles were successfully used in a multi-cycle purification process of the mAb from the CHO feedstock. Boronic acid particles were able to achieve an average overall yield of 86% with 88% removal of CHO host cell proteins (HCP) when the binding was performed at pH 7.4, while at pH 8.5 these values were 58% and 97%, respectively. In both cases, genomic DNA removal was in excess of 97%. Comparatively, Protein A particles recorded an average overall yield of 80% and an HCP removal greater than 99%. The adsorption of the mAb to the boronic acid particles was shown to be mediated by strong affinity interactions. Overall, boronic acid based purification processes can offer a cost-effective alternative to Protein A as the direct capturing step from the mammalian cell culture.

1. Introduction

Monoclonal antibodies (mAbs) play an important role in the treatment of different disorders such as cancer, transplant rejection or auto-immune diseases [1]. This fast growing market valued at $38 billion in 2009 [2] is expected to reach $67.6 billion in sales by 2015 [3]. Currently, there are 34 mAbs approved by the US Food and Drug Administration (http://www.fda.gov/) and many more are still in clinical trials. Unlike other therapeutic proteins or small molecules (e.g. erythropoietin or human growth hormone) mAbs are typically administered at much higher doses due to their relatively low potency [4]. The combination of high doses, large patient populations and treatment of chronic diseases leads to the necessity for large quantities of mAbs to be produced. The ever increasing demand for these therapeutic agents further substantiates the need to establish feasible and economical processes. All of the currently marketed therapeutic antibodies are produced in mammalian cell cultures (Chinese Hamster Ovary (CHO), NS0, Sp2/0, PER.C6), the majority of which by CHO cells [5]. Considering the recent improvements in cell culture productivity, with antibody titers of 27 g/l being reported [6], the focus has now shifted toward the downstream processing. Although many purification strategies can be envisioned, the purification of mAbs follows a rather fixed template in which a capturing step with Protein A affinity chromatography is performed. Protein A is a bacterial cell wall protein from *Staphylococcus aureus* targeting mainly the Fc region of mAbs. Given its high selectivity, purities greater than 98% are typically achieved in a single step from a clarified complex cell culture media [1]. Furthermore, the process is robust, easy to implement (almost no optimization for new products) and applicable to various IgG isoforms [7]. The major drawbacks of this chromatographic step are the high associated costs, which can represent up to 70% of the total downstream costs [8], and the intrinsic limitations of the ligand. The theoretical maximum dynamic binding capacity (DBC) for Protein A has been estimated to be approximately 70 g/l but current chromatographic supports typically achieve 30 g/l [9]. Even if the DBC was to increase to 45 g/l, the maximum mAb titer able to be processed would be 5 g/l [7]. Therefore, cell culture supernatants containing mAb titers in excess of 10 g/l cannot be directly processed by Protein A chromatography, resulting in increased process times with more chromatographic cycles per production cycle. In an attempt to increase throughput while decreasing the overall costs, several different purification strategies have been proposed. Aqueous two-phase systems [10], expanded bed

* Corresponding author. Tel.: +351 218419065; fax: +351 218419062.
E-mail address: rabarros@ist.utl.pt (M.R. Aires-Barros).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2012.07.014

chromatography [11], membrane chromatography [12] and magnetic separations [13] have shown their potential to substitute packed bed chromatography in the purification of mAbs from complex media. Furthermore, all of the aforementioned processes share the potential to enable process integration with resulting benefits in cost reduction and throughput.

Magnetic separations take advantage of the highly specific nature of magnetism to provide fast, gentle and very selective purification processes. Like chromatographic supports, the magnetic carriers can be functionalized with the desired ligand (e.g. affinity, electrostatic, hydrophobic) in order to interact with the target molecule. However, given their usual small size (<2 μm), the particles possess high specific surface areas which can potentially lead to high adsorption capacities. For instance, using negatively charged magnetic particles as ionic exchangers, Ditsch et al. [14] were able to adsorb 640 mg cytochrome c per gram of particle. Magnetic separations have already shown their potential in the purification of lectins from jack bean extracts [15], IgG from human serum [16], IgE from allergic patient sera [17] and mouse IgG from a 100 l CHO cell culture supernatant [13]. The latter further substantiates the potential of magnetic separations at larger scales with significant improvements in process time (only 4 h were required) while achieving identical yield and purity to the conventional chromatographic process.

The present work aims at evaluating the potential of boronic acid as an alternative ligand to Protein A in the direct purification of a human mAb from a CHO cell culture supernatant. The boronic acid ligand is capable of selectively capturing *cis*-diol containing molecules, such as carbohydrates and glycoproteins, through the formation of a reversible covalent ester bond. Antibodies are glycoproteins as they bear oligosaccharides in both the Fc and Fv regions. In the former, despite some heterogeneity, the 1,2-*cis* diol saccharides fucose, manose and galactose can be typically found. From previous work, we have shown that boronic acid functionalized magnetic particles were able to adsorb significant amounts of human IgG (0.216 g IgG/g support) from an IgG aqueous solution [18]. Under typical conditions observed in mammalian cell cultures, best adsorption capacities and less competitive effects by glucose and lactate were observed at pH 7.4. In the present work we shall evaluate the selectivity of the boronic acid particles and fully characterize the purified fractions in terms of product yield and purity. Special attention shall be given to the elucidation of the binding mechanism. To the best of our knowledge this is the first report of magnetic particles functionalized with a synthetic ligand being used to directly process a real CHO cell culture supernatant.

2. Materials and methods

2.1. Biologicals

A CHO cell culture clarified feedstock containing a human IgG1 monoclonal antibody was produced and supplied by ExcellGene (Monthey, Switzerland). An ExcellGene proprietary serum-free medium, containing only one protein, was used for production. Phenol red has been added to the medium as a pH indicator. The CHO cell culture feedstock was initially characterized and was determined to have a mAb concentration of 37 mg/l, an HCP concentration of 61 mg/l (1.66 g HCP/g mAb) and a gDNA concentration of 0.24 mg/l (6.4 gDNA mg/g mAb). The isoelectric point of the mAb was determined using a PhastSystem electrophoresis apparatus from Pharmacia (Uppsala, Sweden). The mAb was initially purified from the CHO cell culture feedstock in a Äkta Purifier system (Amersham Biosciences, Uppsala, Sweden) using a HiTrap MabSelect 1 ml column (GE Healthcare, Uppsala, Sweden). The purified sample was then applied to an isoelectric focusing gel with a pH gradient from 3 to 9 (PhastGel IEF 3-9) and stained with Coomassie Blue PhastGel R-350 both from GE Healthcare (Uppsala, Sweden). The mAb exhibited a broad pI range starting at 6.85 and going up to 8.45. A main variant could not be identified but the highest intensity was observed around pH 8.15. Further characterization of the CHO feedstock was performed by quantifying the concentration of glucose and lactate, metabolites which are known to interact with boronic acid. Using a 7100 MBS Multiparameter Bioanalytical System from YSI Life Sciences (Yellow Springs, OH, USA) the concentrations of glucose and lactate were determined to be 1.15 g/l and 1.48 g/l, respectively.

2.2. Chemicals

SiMAG-Boronic acid and SiMAG-Protein A magnetic particles, consisting of a 10 mg/ml solution of 1 μm non-porous silica beads in water, were acquired from Chemicell (Berlin, Germany). The density of boronic acid ligands was estimated to be 70 μmol/g support according to the binding of alizarin red S as described by Kuzimenkova et al. [19]. All other chemicals were of analytical grade.

2.3. mAb purification from CHO cell feedstock

All the incubation steps were performed at room temperature in a VWR Digital Vortex Mixer at 1000 rpm (Leuven, Belgium). The magnetic particles were separated with a DynaMag^{TM}-2 magnetic particle concentrator from Invitrogen Dynal AS (Oslo, Norway). In a typical experiment, 0.7 mg of previously equilibrated particles (incubated 3 times with 700 μl of binding buffer for 5 min) were incubated for 10 min with 700 μl of CHO cell culture feedstock. The supernatant was recovered and the particles were afterwards washed 3 times with 700 μl of binding buffer (5 min incubation period per wash) in order to remove weakly bound molecules. The adsorbed mAb was recovered in 3 steps by incubating for 5 min each with 700 μl of elution buffer. The binding buffer in all the experiments was 1.5 g/l $NaHCO_3$ 6.4 g/l NaCl corrected to the desired pH. Elution samples from SiMAG-Protein A magnetic particles were neutralized with 1 M Tris–HCl pH 8.5.

2.4. Analytical methods

2.4.1. IgG quantification

The concentration of IgG was determined with an analytical POROS Protein A affinity column from Applied Biosystems (Foster City, CA, USA), using 50 mM sodium phosphate, 150 mM NaCl at pH 7.4 as binding buffer and 12 mM HCl, 150 mM NaCl as elution buffer. Analyses were performed in an Äkta Purifier system (Amersham Biosciences, Uppsala, Sweden) with an A-900 Autosampler fitted with a 500 μl sample loop. Chromatograms were recorded at 215 nm.

2.4.2. IgG aggregate analysis

The presence of IgG aggregates was evaluated by size exclusion chromatography using a TSKgel SuperSW3000 column from Tosoh Bioscience (Stuttgart, Germany). Samples were analyzed for 25 min in isocratic mode using 50 mM phosphate, 300 mM NaCl at pH 7 as the mobile phase at a flow rate of 0.35 ml/min.

2.4.3. Host cell proteins (HCP)

Host cell protein levels were determined using a CHO Host Cell Proteins 3rd Generation Elisa kit from Cygnus Technologies (Southport, NC, USA). Samples were diluted in Sample Diluent Buffer from the same company. Absorbance measurements were performed on a SpectraMax Plus384 microplate reader from Molecular Devices (Sunnyvale, CA, USA).

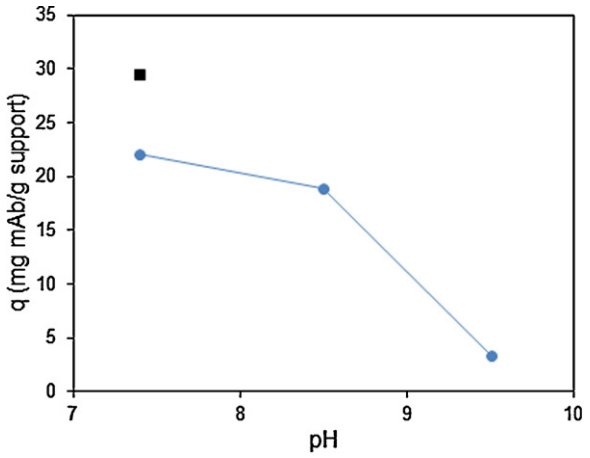

Fig. 1. Binding capacity of SiMAG-Boronic acid (●) and SiMAG-Protein A (■) magnetic particles as function of the CHO cell culture supernatant pH.

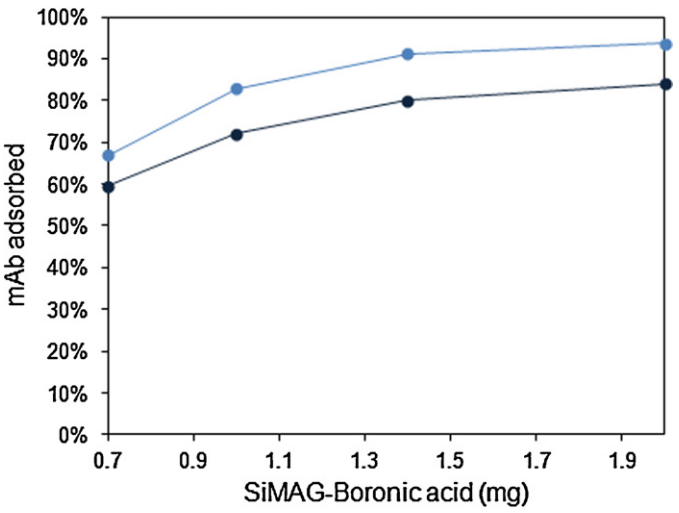

Fig. 2. Effect of the amount of SiMAG-Boronic acid magnetic particles used in the percentage of human mAb adsorbed from 700 μl of a CHO cell culture supernatant at pH 7.4 (●) and 8.5 (●).

2.4.4. CHO genomic DNA (gDNA)

CHO genomic DNA was quantified by real time PCR following a procedure adapted from Nissom [20]. Reactions were carried out in a LightCycler from Roche Applied Science (Manheim, Germany) using the following conditions: pre-incubation at 95 °C for 10 min, followed by 40 cycles each of denaturation at 95 °C for 10 s, annealing at 60 °C for 5 s and extension at 72 °C for 10 s. Two microliters of sample (2 μl) were amplified in a total volume of 20 μl mixture containing LightCycler-FastStart DNA Master SYBR Green I (Roche Applied Science), 0.2 μM of each primer and 3 mM of $MgCl_2$. The primers NV1_F (5′-ACAGGTTTCTGCTTCTGGCT) and NV1_R (5′-CATCAGCTGACTGGTTCACA) were synthesized by STAB Vida (Caparica, Portugal). A calibration curve was produced from CHO genomic DNA extracted using Wizard Genomic DNA isolation kit from Promega (Madison, WI, USA).

2.4.5. Protein gel electrophoresis

Protein compositions were analyzed by reducing SDS-PAGE [21] using 4–12% acrylamide gels. Gels were prepared from 40% acrylamide (AppliChem, Darmstadt, Germany) and 2% N,N′-Methylenebisacrylamide (GE Healthcare, Uppsala, Sweden) stock solutions. Samples were denaturated in reducing conditions with dithiothreitol (100 mM final concentration) at 100 °C for 5 min. Gels were ran at 90 mV and stained with Coomassie Blue PhastGel R-350 (GE Healthcare, Uppsala, Sweden). Images were acquired with a GS-800 calibrated densitometer from Bio-Rad (Hercules, CA, USA).

3. Results and discussion

Several different parameters were evaluated in the optimization of the mAb purification process from a CHO cell culture feedstock, namely the binding pH, sample volume to magnetic particle ratio and elution buffer.

3.1. Effect of binding pH

The effect of the binding pH was evaluated by correcting the pH of the CHO feedstock to 7.4, 8.5 and 9.5. Prior to the adsorption step the particles were equilibrated with 1.5 g/l $NaHCO_3$ 6.4 g/l NaCl at the appropriate pH. Fig. 1 shows the effect of the CHO feedstock pH in the binding capacity of SiMAG-Boronic acid and SiMAG-Protein A magnetic particles. As it can be observed, the binding capacity of SiMAG-Boronic acid particles was highest at pH 7.4 (22 mg mAb/g support) and sharply decreased as the pH increased from 8.5 to 9.5. In fact, at pH 9.5 less than 15% of the mAb was adsorbed while at pH 7.4 and 8.5 this value was 69% and 59%, respectively. Comparatively, SiMAG-Protein A particles at pH 7.4 showed a binding capacity of 29 mg mAb/g support and were able to adsorb 91% of the mAb present. The lower capacity of the boronic acid particles is mainly due to the presence of competing species, namely glycosylated host cell proteins and small *cis*-diol or α-hydroxy acid molecules (e.g. glucose, lactate) in the cell culture feedstock. As we previously reported in a study performed with pure polyclonal human IgG, the binding pH plays a major role in the profile of the adsorption isotherms and in the binding capacity of the support in the presence of competing *cis*-diols and α-hydroxy acids [18]. While the adsorption isotherms with pure polyclonal human IgG at pH 7.4 and 8.5 were identical, at pH 9.5 the maximum binding capacity decreased approximately 25% and the support revealed a lower affinity toward IgG (lower initial slope) [18]. However, when comparing the binding capacities of SiMAG-Protein A and SiMAG-Boronic acid particles at both pH 7.4 and 8.5 for low antibody titers as in the CHO feedstock used in this work, 37 mg/l, they were identical. Therefore, as previously mentioned, the differences observed are mainly due to the presence of competing species.

To further demonstrate the effect of the competing metabolites the CHO supernatant was concentrated and dialyzed with binding buffer (1.5 g/l $NaHCO_3$ and 6.4 g/l NaCl) at pH 7.4 and 8.5 using a 3 kDa cut-off membrane. In both cases, the binding capacity increased sharply with approximately 98% of the mAb being adsorbed thus proving the competing effect of the metabolites present in the CHO supernatant.

3.2. Effect of magnetic particle loading

The effect of sample volume to magnetic particle mass ratio was varied to determine the conditions that promoted the highest adsorption yields. Considering the low binding capacities previously observed at pH 9.5, these studies were only conducted at pH 7.4 and 8.5. Starting with a fixed volume of CHO feedstock (700 μl) the mass of SiMAG-Boronic acid particles was varied from 0.7 mg to 1, 1.4 and 2 mg. Fig. 2 shows the effect of the amount of SiMAG-Boronic acid particles in the adsorption of the mAb from the CHO feedstock at pH 7.4 and 8.5. For both pH values, the trend observed was identical, with the percentage of mAb adsorbed reaching a plateau for an adsorbent mass greater than 1.4 mg. Furthermore, the percentage difference between the values registered for both pH values was found to be fairly constant (approximately 10%) with lower values being obtained at pH 8.5. This difference can

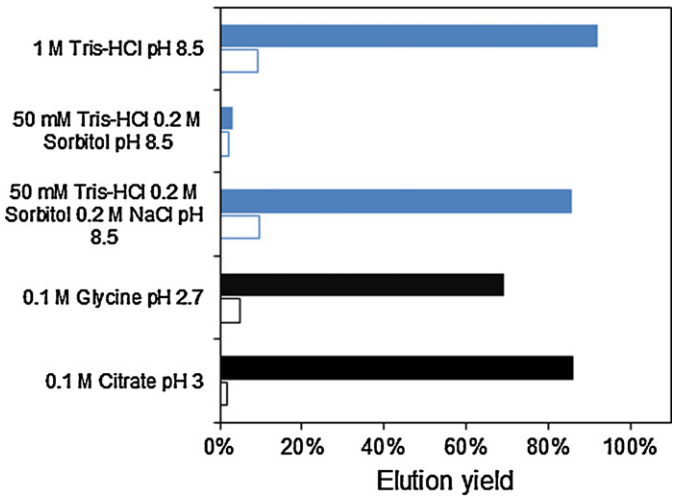

Fig. 3. Human mAb recovery yield in the first (solid bars) and second (empty bars) elution fractions at various elution conditions for SiMAG-Boronic acid ( ) and SiMAG-Protein A (■) magnetic particles.

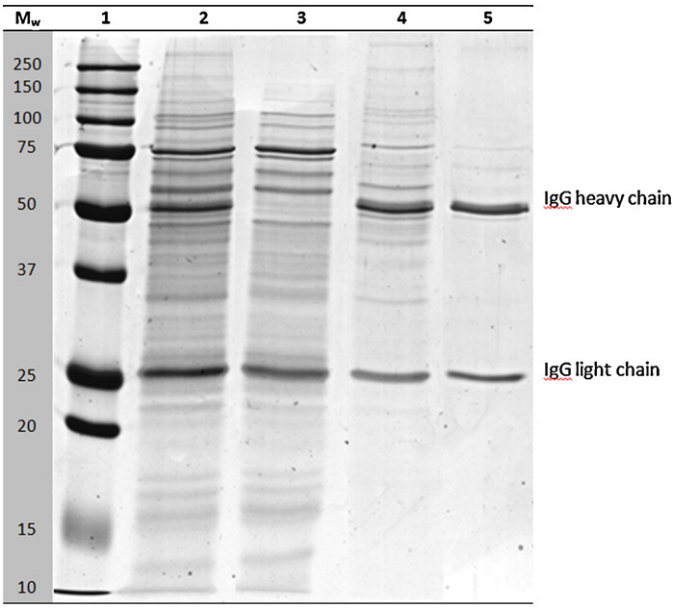

Fig. 4. Reducing SDS-PAGE analysis of the effect of the binding pH in the elution fraction purity from SiMAG-Boronic acid particles. Lane 1 – protein molecular weight (kDa) standards, Lane 2 – CHO feedstock, Lane 3 – adsorption supernatant, Lane 4 – elution fraction after adsorption at pH 7.4, Lane 5 – elution fraction after adsorption at pH 8.5.

indicate the presence of non-specific interactions with boronic acids, namely through charge transfer, which tend to decrease as the pH is closer to the pKa of the ligand (8.8 for phenylboronic acid) [22]. Conversely, at lower pH values non-specific interactions are more predominant as it has been shown by several studies through the ability of boronic acid ligands to bind non-glycosylated proteins [23–28]. The use of 1.4 mg of adsorbent appears to be a good compromise allowing the adsorption of 91% and 80% of the mAb at pH 7.4 and 8.5, respectively. The impossibility to reach 100% adsorption at both pH values can suggest that a small mAb population is either lacking glycosylation or exhibiting a different glycosylation pattern without *cis*-diol glycans. The glycan structure of antibodies is very complex and it is affected by cell culture process variables, media composition and the metabolic pathways of the producing clone [29]. Even in the production of carefully controlled therapeutic monoclonal antibodies an heterogeneous population is observed [30]. As reported by Lim et al. [31], non-glycosylated Fc variants of mAbs might be produced by some CHO clones. Furthermore, the way carbohydrates are linked to one another affect the availability of *cis*-diol groups. For instance in the G0F glycoform of the N-linked glycan in the Fc region of antibodies produced by CHO cells, the terminal N-acetylglucosamine might be linked to mannose through 1–2 [32] or 1–4 [30] glycosidic linkages with only the latter exhibiting a *cis*-diol moiety. Considering all these possibilities it is normal that not all antibodies possess *cis*-diol moieties for the affinity interaction with the boronic acid ligand.

3.3. *Effect of elution conditions*

Several elution buffers were selected based on previous results [18] and evaluated in order to determine the conditions that provided better yield and purity. For SiMAG-Boronic acid particles the elution buffers tested were: (i) 1 M Tris–HCl pH 8.5; (ii) 50 mM Tris–HCl, 200 mM sorbitol, pH 8.5 and (iii) 50 mM Tris–HCl, 200 mM sorbitol, 200 mM NaCl, pH 8.5. For SiMAG-Protein A particles, two elution buffers were evaluated: (i) 0.1 M citrate pH 3 and (ii) 0.1 M glycine pH 2.7. In order to ensure the removal of non-specifically bound proteins, the particles were washed 3 times with binding buffer after the incubation step with the CHO feedstock. In any case, these fractions showed neither impurities nor mAb (through SDS-PAGE and Protein A HPLC analysis), revealing that the proteins were tightly bound to the particles. Product recovery was later on promoted by three sequential elution steps. Fig. 3 shows the mAb recovery yield in the first and second elution fractions for the elution buffers tested for both SiMAG-Boronic acid and SiMAG-Protein A magnetic particles. As it can be observed, regardless of the elution conditions employed the vast majority of the mAb recovered was obtained in the first elution fraction (>90%) while only a small amount was eluted in the second fraction (<10%). No antibody was present in the third elution fraction. Comparing all the results, 1 M Tris–HCl pH 8.5 and 0.1 M citrate pH 3 were found to provide the best results for SiMAG-Boronic acid and SiMAG-Protein A particles, respectively. In order to evaluate the potential of achieving product concentration, the elution was carried out at half of volume typically employed. Identical recovery yields were observed when eluting the mAb in 350 μl of elution buffer, thus validating this strategy. Interestingly, given the intrinsic proprieties of the mAb the best elution conditions differed from our previous findings with polyclonal human antibody [18]. In the present work, the best results were achieved with 1 M Tris–HCl pH 8.5 and the 50 mM Tris–HCl, 200 mM sorbitol pH 8.5 elution buffer that was able to recover more than 80% of the polyclonal human antibody, in our previous study, was not able elute the mAb. Such is due to the lower pI of the mAb in comparison with the polyclonal antibody used, which results in the adsorption of the former to the particles through electrostatic interactions in the absence of NaCl in the elution buffer (positive particles and overall negative antibody).

In terms of product purity, the elution fractions of SiMAG-Boronic acid particles were most influenced by the binding pH rather than the elution buffer itself. In fact, regardless of the elution buffer used, the SDS-PAGE profile of the elution fractions was always the same. Fig. 4 shows the SDS-PAGE analysis of the first elution fractions from SiMAG-Boronic acid particles in which the binding was performed at pH 7.4 and 8.5 as well as the CHO feedstock and the adsorption supernatants (unbound fraction). As it can be observed, greater purities were achieved when the binding was performed at pH 8.5 (Lane 5), revealing an almost pure fraction. The presence of more impurities when the mAb adsorption was promoted at pH 7.4 (Lane 4) is consistent with the aforementioned higher potential of the boronic acid ligand to promote non-specific interactions at pH values lower than its pKa. Nevertheless, when

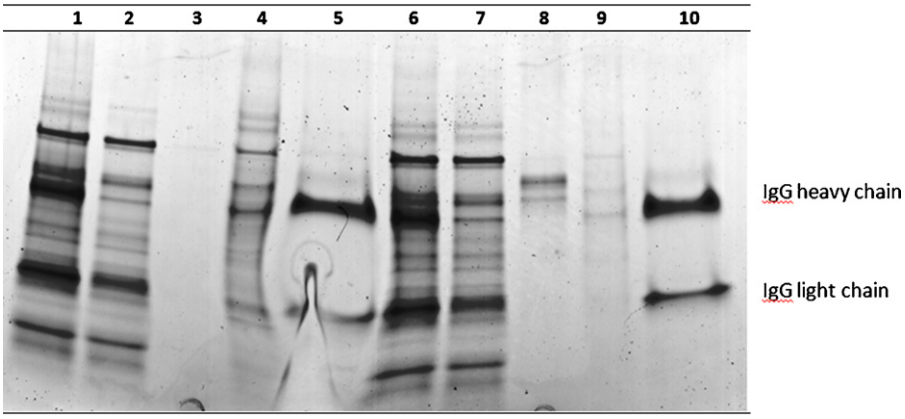

Fig. 5. Reducing SDS-PAGE analysis of the effect of 6 M urea in the elution of the mAb from SiMAG-Boronic acid particles. Binding performed at pH 7.4 (Lane 1–5) and 8.5 (Lane 6–10). Lane 1 and 6 – CHO feedstock, Lane 2 and 7 – adsorption supernatant, Lane 3 and 8 – washing fraction, Lane 4 and 9 – elution fraction with 6 M urea, Lane 5 and 10 – elution fraction with 1 M Tris–HCl pH 8.5.

comparing both elution fractions with the starting CHO feedstock (Lane 2), we can observe that boronic acid particles were able to selectively adsorb the mAb and thus provide a great improvement in purity. Moreover, when comparing the CHO feedstock (Lane 2) with the corresponding adsorption supernatant (Lane 3), it is evident that the selectivity of the process is rendered by the selectivity of the adsorption step rather than being achieved by the selective elution of the mAb and/or by the removal of impurities through different washing steps. This behavior is remarkably different from several multimodal ligands aimed at serving as an alternative to the Protein A capturing step, such as 4-mercapto-ethyl-pyridine (MEP) [33] and hexylamine (HEA) [34], where the optimization of the washing and elution steps is crucial to obtain suitably pure products. In addition, the low selectivity of the adsorption step typically results in a sharp decrease in binding capacity toward the target molecule, in a higher probability of having irreversibly bound proteins and thus in the necessity of having a stricter control in the support regeneration process. A testament to the high selectivity of the SiMAG-Boronic acid supports comes from the previous section (Section 3.2) when the ratio of adsorbent to CHO feedstock volume was varied. In this case, regardless of the ratio used no differences in the SDS-PAGE purity of the elution fractions was observed thus showing that the increased binding surface did not promote a higher degree of non-specific binding.

3.4. Elucidation of the binding mechanism

The phenylboronic acid ligand can be viewed as a multi-modal ligand as it is able to promote a multitude of interactions, namely, affinity, electrostatic, hydrophobic, aromatic π–π, charge transfer and hydrogen bonding. Depending on the conditions, non-affinity interactions, i.e. secondary interactions, might predominate. As the present work is aimed at the direct capturing of mAbs from a CHO supernatant the initial conditions, with the exception of the pH, are set. Therefore, the presence of different secondary interactions was evaluated by the ability of recovering the product with the corresponding competitors. In these experiments, the mAb was adsorbed to the particles at pH 7.4 and 8.5 and subsequently washed with binding buffer. Afterwards, the particles were incubated with the corresponding test buffer (350 μl) for 5 min and subsequently with 1 M Tris–HCl pH 8.5 (350 μl).

In order to screen for hydrophobic interactions the particles were incubated with 20 mM HEPES pH 7. The choice these conditions resides in the fact that HEPES is not a *cis*-diol competitor and at this pH value the SiMAG-Boronic acid particles are positively charged (16 mV in 10 mM KNO_3 pH 7 [18]) while the mAb is either neutral or positively charged, thus, ensuring that no electrostatic interactions are promoted. Less than 1.5% of IgG was recovered showing that hydrophobic interactions are negligible. In order to

Fig. 6. (A) Overall yield in the multi-cycle purification of a human mAb from a CHO feedstock using SiMAG-Protein A particles (■) and SiMAG-Boronic acid particles with the adsorption step at pH of 7.4 (●) and 8.5 (●). The overall yield was calculated considering solely the first elution fraction in each cycle. (B) Host cell protein removal (solid symbols) and purification factor (empty symbols) in the multi-cycle purification of a human mAb from a CHO feedstock using SiMAG-Protein A particles (■) and SiMAG-Boronic acid particles with the adsorption step at pH of 7.4 (●) and 8.5 (●). Values represented correspond solely to the first elution fraction of each cycle.

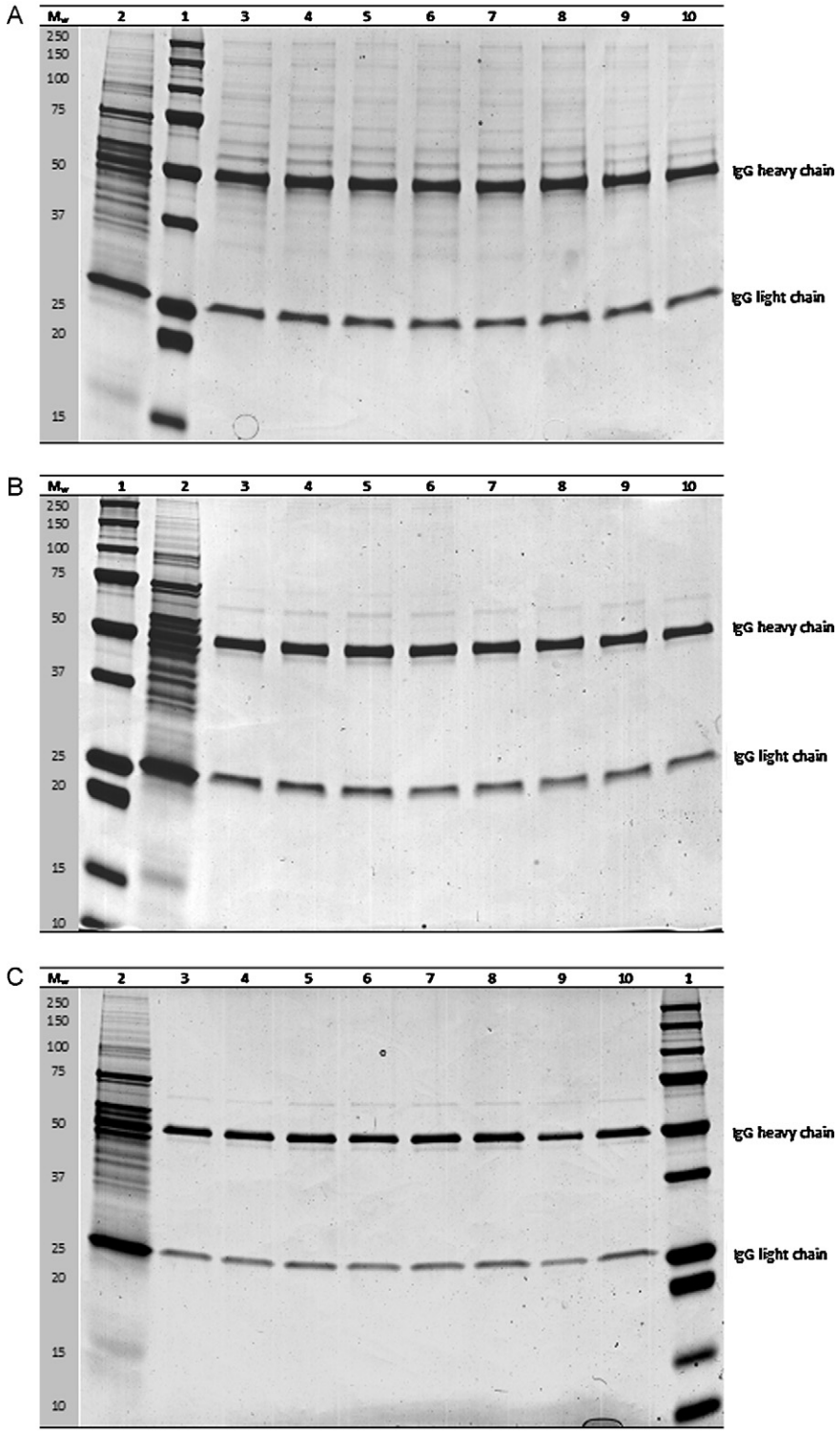

Fig. 7. Reducing SDS-PAGE analysis of the multi-cycle purification of a human mAb from a CHO feedstock using: (A) SiMAG-Boronic acid with the adsorption step at pH 7.4, (B) SiMAG-Boronic acid with the adsorption step at pH 8.5, (C) SiMAG-Protein A. Lane 1 – protein molecular weight (kDa) standards, Lane 2 – CHO feedstock, Lane 3–10 – first elution fraction of cycle 1–8.

screen for electrostatic interactions, the particles were incubated with binding buffer supplemented with 1 M NaCl, at pH 7.4 and 8.5. In this case, the amount of IgG recovered was always lower than 7%. Slightly higher recoveries were obtained at pH 8.5, as electrostatic effects can be present since a higher amount of the boronic acid ligand is in its anion form. Nevertheless, the difference was marginal (approximately 2%). In all cases, the product was largely subsequently recovered with the *cis*-diol competitor, corroborating a strong and specific affinity interaction. Strong non-specific interactions were still screen by incubating the particles with 6 M urea, as a couple of reports have shown the ability of recovering boronic acid bound erythropoietin [35] and the heavy chain of a monoclonal antibody [36] using high concentrations of urea. Fig. 5 shows the SDS-PAGE profile of the various fractions. As it can be observed no

Table 1
Summary of the results obtained for the multi-cycle purification of a human mAb from a CHO feedstock using SiMAG-Boronic acid and SiMAG-Protein A particles. The values presented are averages of the 8 cycles. The overall yield was calculated considering only the first elution fraction in each cycle. The purification factor (PF) was defined as the ratio of HCP/mAb in each elution fraction and in the starting CHO feedstock.

	Adsorption pH	IgG (mg/l)	HCP (mg/l)	gDNA (ng/ml)	HCP removal	PF	gDNA removal	CF	Overall yield
CHO supernatant	–	37	61	236					
SiMAG-Boronic acid	pH 7.4	64	15	<15	88.0%	7.4	>97%	1.72	86.1%
	pH 8.5	43	4	<15	97.1%	20.0	>97%	1.17	58.3%
SiMAG-Protein A	pH 7.4	59	0.6	N.D.	99.5%	174.9	N.D.	1.60	80.0%

CF: concentration factor.
N.D.: not determined.

significant amounts of IgG were recovered with 6 M urea (Lane 4 and 9) regardless of the initial binding pH used (pH 7.4 and 8.5). Once more, the product was afterwards efficiently recovered with the *cis*-diol competitor thus showing that the affinity interaction predominates. Interestingly, at pH 7.4 the use of 6 M urea allowed for the removal of almost all bound protein impurities rendering an almost pure elution fraction with 1 M Tris–HCl pH 8.5. Nevertheless, this washing strategy was not employed in the studies as the effect of urea in the antibody could not be evaluated.

3.5. Multi-cycle purification

The re-usability of the particles was tested by performing 8 purification cycles. Each cycle consisted of (i) equilibration of the particles 3 times with 700 μl of binding buffer; (ii) 10 min incubation with 700 μl of CHO feedstock corrected to the desired pH; (iii) 5 min washing with 700 μl of binding buffer; and (iv) elution by incubating the particles 2 times with 350 μl of elution buffer for 5 min. Considering the results previously described, the mAb purification with SiMAG-Boronic acid particles was performed with 1.4 mg of adsorbent while the elution was carried out with 1 M Tris–HCl pH 8.5. As for SiMAG-Protein A particles, 1 mg of adsorbent was used and the elution performed with 0.1 M citrate pH 3. For the determination of the overall yield, only the first elution fraction was considered since it contained more than 90% of the total eluted product. Furthermore, since the elution volume was half of the initial CHO feedstock volume, we were thus able to achieve product concentration. Fig. 6 A shows the overall yield in each purification cycle using SiMAG-Protein A and SiMAG-Boronic acid particles. As it can be observed, at both pH values, SiMAG-Boronic acid particles had an identical trend with a slight decrease in the overall yield as the number of cycles increased. Regardless of the binding pH, the elution yield was found to be identical throughout the purification cycles and all bound mAb was recovered in the two elution steps. As the binding capacity was found to decrease slightly throughout the process and it was visually observed that the magnetic particles tended to adhere to the plastic pipette tips, the decrease in the overall yield was most probably due to the loss of adsorbent throughout the cycles.

In regard to SiMAG-Protein A particles, the behavior was slightly different. The binding capacity was the same throughout the 8 cycles and the particles were not found to adhere to the plastic pipette tips. Fig. 6 B shows the HCP removal and associated purification factor for each purification cycle. For a clear visual analysis reducing SDS-PAGE are provided in Fig. 7. In terms of HCP removal values greater than 83% were always achieved with the worst results being attained with SiMAG-Boronic acid particles at pH 7.4. Comparatively, at pH 8.5 this value was greater than 96% which was slightly less than Protein A at 99.5% HCP removal. The variation in HCP removal throughout the purification cycles was very constant for SiMAG-Boronic acid particles at pH 8.5 and SiMAG-Protein A particles at pH 7.4. Conversely, for SiMAG-Boronic acid particles at pH 7.4 a slight increase in HCP removal was observed reaching 94% in the last purification cycle. These values can be confirmed by the reducing SDS-PAGE analysis of the elution fractions. As it can be observed in Fig. 7A, the electrophoresis profile of the elution fractions of the SiMAG-Boronic acid particles at pH 7.4 was identical in all of the 8 cycles but with less intense impurity bands especially in the last 2 cycles. Interestingly, the reducing SDS-PAGE profile of SiMAG-Boronic acid particles at pH 8.5 (Fig. 7 B) and SiMAG-Protein A (Fig. 7C) was almost the same with both exhibiting a faint additional impurity band around 75 kDa. The improvement in product purity can be quantified by the purification factor defined as the mAb/HCP ratio of the elution fractions with the value observed in the initial feedstock. Given the very high HCP removal, SiMAG-Protein A particles were able to achieve approximately a 175 fold increase in each cycle without significant variations (Fig. 6B). Comparatively, SiMAG-Boronic acid particles at pH 8.5 and 7.4 also showed few variations throughout the purification cycles but this value dramatically decreased to 20 and 7 fold, respectively. In Table 1 is represented the summary of the average results for the different purification processes. SiMAG-Boronic

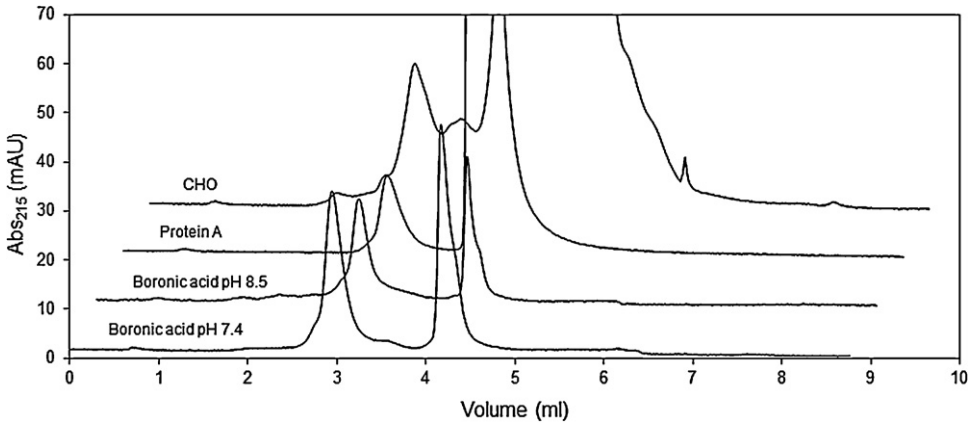

Fig. 8. Size exclusion chromatography analysis of the CHO feedstock and elution fractions from SiMAG-Protein A and SiMAG-Boronic acid particles at pH 7.4 and 8.5.

acid particles at pH 7.4 provided the best average overall yield with 86.1% while providing a concentration factor of 1.72 (mAb concentration in the elution fractions of 64 mg/l). While these results were better than those of SiMAG-Protein A at the same pH with 80.0% and 1.60, respectively, boronic acid particles were outperformed in terms of HCP removal achieving 88% against 99.5%. Comparatively, SiMAG-Boronic acid particles at pH 8.5 were able to achieve a 97.1% HCP removal but with the disadvantage of only providing a 58.3% overall yield. As an initial capturing step, higher yields are preferred to higher purities. Therefore, the purification process with SiMAG-Boronic acid particles at pH 7.4 provides a more suitable alternative than the process at pH 8.5 to substitute Protein A as the capturing step. In addition to the HCP removal, both boronic acid based processes were able to achieve at least a 97% reduction in genomic DNA. The gDNA concentration in SiMAG-Protein A elution samples was not able to be quantified due to the presence of citrate and concomitant chelation of the required Mg^{2+} for the DNA amplification. Sample dilution was not feasible as it would decrease the DNA concentration to a value below the detection limit. Nevertheless, when analyzing the adsorption supernatants identical gDNA concentrations of those of SiMAG-Boronic acid particles were found. Therefore, a gDNA removal in excess of 97% is also expected for SiMAG-Protein A magnetic particles.

Fig. 8 shows the size exclusion chromatograms of the CHO feedstock and the elution fractions from SiMAG-Protein A and SiMAG-Boronic acid particles at pH 7.4 and 8.5. As it can be observed, the elution fractions do not show aggregates since there is no peak eluting before the mAb (retention volume 2.94 ml).

4. Conclusion

Boronic acid magnetic particles were successfully used in the direct capture of a human mAb from a CHO feedstock. The process showed to be highly selective and provided high clearance of process impurities such as HCP (>83%) and gDNA (>97%). Among the process parameters evaluated, the pH of the adsorption step was found to be the most preponderant for both purity and yield. While at pH 7.4 higher yields were achieved, greater purities were observed at pH 8.5. Comparatively to Protein A magnetic particles, boronic acid particles at pH 7.4 provided a 6% higher yield while achieving only 12% less in HCP removal. Furthermore, the purification strategies were shown not to promote product aggregation. Given the remarkably lower costs of this synthetic ligand, its non-toxic proprieties and higher chemical stability, it can be an interesting alternative to Protein A in order to reach a more cost effective purification process. In addition, as the boronic acid ligand recognizes specifically the glycan structure of the antibody it is possible, unlike with Protein A, to purify antibody fragments lacking the Fc region provided that they are glycosylated in its Fv region. As an alternative to conventional packed-bed systems, we have shown that magnetic separations are able to provide the desired purification while potentially providing faster processes conducted under very gentle conditions. Furthermore, the particles were shown to be robust rendering identical results in 8 consecutive purification cycles.

Acknowledgements

L. Borlido acknowledges Fundação para a Ciência e Tecnologia (FCT) for the Ph.D. fellowship BD/45077/2008. A. M. Azevedo acknowledges the initiative “Ciência 2007” of the Portuguese Ministry for Science, Technology, and Higher Education (http://www.mctes.pt/). The authors would also like to acknowledge FCT for the financial support through the contract *PTDC/EBB-BIO/098961/2008*.

References

[1] A.A. Shukla, J. Thömmes, Trends Biotechnol. 28 (2010) 253.
[2] B.J. Horstmann, H.A. Chase, Chem. Eng. Des. 67 (1989) 243.
[3] BIResearch. The Future of Monoclonal Antibodies - Market Forecasts to 2015, Competitive Benchmarking, Product Pipeline and Deals Analysis. December 1, 2009 [cited 2012 March 29]; Available from: http://www.marketresearch.com/product/display.asp?productid=2515126.
[4] S. Aldington, J. Bonnerjea, J. Chromatogr. B 848 (2007) 64.
[5] B. Kelley, MAbs 1 (2009) 443.
[6] M. Kuczewski, E. Schirmer, B. Lain, G. Zarbis-Papastoitsis, J. Biotechnol. 6 (2011) 56.
[7] M. Trexler-Schmidt, S. Sze-Khoo, A.R. Cothran, B.Q. Thai, S. Sargis, B. Lebreton, B. Kelley, G.S. Blank, BioPharm International, 2009, p. 8.
[8] A.M. Azevedo, P.A.J. Rosa, I.F. Ferreira, M.R. Aires-Barros, Trends Biotechnol. 27 (2009) 240.
[9] D. Low, R. O'Leary, N.S. Pujar, J. Chromatogr. B 848 (2007) 48.
[10] W. Bäcker, S., Sommerfeld, M., Mutter, P.A.J., Rosa, M.R., Aires-Barros, A.M. Azevedo, in: WIPO Patent 112149, 2009, September 17.
[11] R.L. Fahrner, G.S. Blank, G.A. Zapata, J. Biotechnol. 75 (1999) 273.
[12] C. Boi, J. Chromatogr. B 848 (2007) 19.
[13] K. Holschuh, A. Schwämmle, J. Magn. Magn. Mater. 293 (2005) 345.
[14] A. Ditsch, J. Yin, P.E. Laibinis, D.I.C. Wang, T.A. Hatton, Biotechnol. Progr. 22 (2006) 1153.
[15] A. Heebøll-Nielsen, M. Dalkiær, J.J. Hubbuch, O.R.T. Thomas, Biotechnol. Bioeng. 87 (2004) 311.
[16] H. Qian, C. Li, Y. Zhang, Z. Lin, J. Immunol. Methods 343 (2009) 119.
[17] T.C.A. Zollner, R.d.L. Zollner, M. De Cuyper, M.H.A. Santana, J. Dispers. Sci. Technol. 24 (2003) 615.
[18] L. Borlido, A.M. Azevedo, A.C.A. Roque, M.R. Aires-Barros, J. Chromatogr. A 1218 (2011) 7821.
[19] M.V. Kuzimenkova, A.E. Ivanov, C. Thammakhet, L.I. Mikhalovska, I.Y. Galaev, P. Thavarungkul, P. Kanatharana, B. Mattiasson, Polymer 49 (2008) 1444.
[20] P.M. Nissom, Biologicals 35 (2007) 211.
[21] U.K. Laemmli, Nature 227 (1970) 680.
[22] X.-C. Liu, W.H. Scouten, Boronate affinity chromatography, in: P. Bailon, G.K. Ehrlich, W.-J. Fung, W. Berthold (Eds.), Affinity Chromatography, Humana Press, 2000, p. 119.
[23] B.M. Brena, F. Batista-Viera, L. Rydén, J. Porath, J. Chromatogr. A 604 (1992) 109.
[24] A.G. Gomes, A.M. Azevedo, M.R. Aires-Barros, D.M.F. Prazeres, J. Chromatogr. A 1217 (2010) 2262.
[25] F.A. Middle, A. Bannister, A.J. Bellingham, P.D. Dean, Biochem. J. 209 (1983) 771.
[26] F.B. Viera, B.B. Barragan, B.L. Busto, Biotechnol. Bioeng. 31 (1988) 711.
[27] A.M. Azevedo, A.G. Gomes, L. Borlido, I.F.S. Santos, D.M.F. Prazeres, M.R. Aires-Barros, J. Mol. Recognit. 23 (2010) 569.
[28] A.G. Gomes, A.M. Azevedo, M.R. Aires-Barros, D.M.F. Prazeres, J. Chromatogr. A 1218 (2011) 8629.
[29] P. Hossler, S.F. Khattak, Z.J. Li, Glycobiology 19 (2009) 936.
[30] J. Qian, T. Liu, L. Yang, A. Daus, R. Crowley, Q. Zhou, Anal. Biochem. 364 (2007) 8.
[31] A. Lim, A. Reed-Bogan, B.J. Harmon, Anal. Biochem. 375 (2008) 163.
[32] A. Beck, E. Wagner-Rousset, M.-C. Bussat, M. Lokteff, C. Klinguer-Hamour, J.-F. Haeuw, L. Goetsch, T. Wurch, A.V. Dorsselaer, N. Corvaia, Curr. Pharm. Biotechnol. 9 (2008) 482–501.
[33] K. Sparbier, T. Wenzel, M. Kostrzewa, J. Chromatogr. B 840 (2006) 29.
[34] W.-C. Lee, C.-Y. Chuang, J. Chromatogr. A 721 (1996) 31.
[35] D. Zanette, A. Soffientini, C. Sottani, E. Sarubbi, J. Biotechnol. 101 (2003) 275.
[36] B. Zhang, S. Mathewson, H. Chen, J. Chromatogr. A 1216 (2009) 5676.