Aminophenyl boronic acids can form reversible covalent ester interactions with \(\text{cis}\)-dial-containing molecules, serving as a selective tool for binding glycoproteins as antibody molecules that possess oligosaccharides in both the Fv and Fc regions. In this study, amino phenyl boronic acid (APBA) magnetic particles (MPs) were applied for the magnetic separation of antibody molecules. Iron oxide MPs were firstly coated with dextran to avoid non-specific binding and then with 3-glycidyloxypropyl trimethoxysilane to allow further covalent coupling of APBA (APBA_MP). When contacted with pure protein solutions of human IgG (hIgG) and bovine serum albumin (BSA), APBA_MP bound \(170 \pm 10\) mg hIgG g\(^{-1}\) MP and eluted \(160 \pm 5\) mg hIgG g\(^{-1}\) MP, while binding only \(15 \pm 5\) mg BSA g\(^{-1}\) MP. The affinity constant for the interaction between hIgG and APBA_MP was estimated as \(4.9 \times 10^5\) M\(^{-1}\) (\(K_a\)) with a theoretical maximum capacity of \(492\) mg hIgG adsorbed g\(^{-1}\) MP (\(Q_{\text{max}}\)), whereas control particles bound a negligible amount of hIgG and presented an estimated theoretical maximum capacity of \(3.1\) mg hIgG adsorbed g\(^{-1}\) MP (\(Q_{\text{max}}\)). APBA_MP were also tested for antibody purification directly from CHO cell supernatants. The particles were able to bind \(98\%\) of IgG loaded and to recover \(95\%\) of pure IgG (purity greater than \(98\%\)) at extremely mild conditions.

1. Introduction

Antibodies and antibody-derived molecules represent an important group of biopharmaceuticals, to which several improvements have been reached at the upstream level over the past years [1]. Downstream processes have also been improved with several new non-chromatographic techniques gaining increased relevance, namely aqueous two-phase systems [2], crystallization/precipitation [3] and magnetic fishing [4]. Iron oxide magnetic particles (MPs) find applications as nanomedical vectors [5], carriers for biocatalysis, biomedical applications [6], biosensing purposes [7], magnetofection agents [8], as well as matrices for purification processes [9]. The great interest in these materials, in particular for bioseparation purposes, arises from properties such as superparamagnetism, high surface-to-volume ratio and fast binding kinetics, as well as an extreme versatility for chemical derivatization [10]. Superparamagnetism is the lack of magnetic memory, a property ideally suited for reversible adsorption–desorption processes such as the ones employed in protein purification. Commonly, MPs consist of stable colloidal MPs embedded in a polymer or of polymer-coated iron-oxide crystal clusters. The surface-to-volume ratio is intrinsically correlated with the final hydrodynamic size of the MPs or clusters [11], which can be controlled by tuning MP synthesis and modification [12]. In order to render MPs active towards binding-specific biomolecules, polymer-coated particles can be chemically modified with suitable functional groups such as oleylamine [13], and then with ligand similar to what is done in chromatographic resins [14]. When considering the purification of antibody molecules,
magnetic fishing is particularly suited for an initial capture step, usually conducted with protein-A-functionalized chromatographic resins [15]. There are protein A-coated MPs commercially available for the purification of antibodies, and previous studies indicated that other types of functionalized MPs can also be suitable for the capture step. In particular, our groups have shown that custom-made MPs coated with small and robust synthetic ligands mimicking protein A [16], and commercial boronic acid-coated MPs [17] represent interesting and viable options for capturing antibodies in purification processes. Commercial boronic acid MPs allowed the direct and selective capture of a human mAb from a CHO feedstock, where binding and elution conditions were tuned to reach maximum yield and final purity [17]. However, the high cost of these commercial particles makes them unviable the scale-up of the purification process. Therefore, encouraged by the results obtained with commercial boronic acid particles, this work focused on the development of iron oxide MPs coated with dextran, a polymer already shown to greatly reduce non-specific interactions [16], further chemically modified with aminophenyl boronic acid by three distinct routes, which resulted in the development of an easy and scalable procedure for MP synthesis and subsequent application on the one-step recovery of antibodies.

2. Material and methods

2.1. Materials

The reagents employed were of highest available purity and used without further purification. 3-Glycidoxypropyl trimethoxysilane (GLYMO), ferric sulfate hydrate \( \text{Fe}_2(\text{SO}_4)\cdot3\text{H}_2\text{O} \), ferrous sulfate heptahydrate \( \text{FeSO}_4 \cdot 7\text{H}_2\text{O} \), (3-aminopropyl) triethoxysilane (APTES), sodium silicate \( \text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O} \), amino phenyl boronic acid (APBA), tris(hydroxymethyl)aminomethane, dextran from Leuconostoc mesenteroides, glutaraldehyde, tetraethoxy silane (TEOS), gum arabic, anthrone, sodium hydroxide and alizarin red (AR) were purchased from Sigma-Aldrich. Glycine was purchased from Fluka. Bicinchoninic acid (BCA) kit from Sigma was used for protein quantification assay. The reagents used for SDS–PAGE gels were ammonium persulfate (APS), \( N,N,N,N \)-tetramethylethylenediamine (TEMED), 30% acrylamide/bisacrylamide solution, sodium dodecyl sulfate micropellets, silver stain plus kit purchased from Bio-Rad; the proteins employed were human immunoglobulin from Octapharma (95%) and albumin from bovine serum (98%) purchased from Sigma-Aldrich. All the spectrofluorometric measurements were taken with the microplate reader Infinite F200 from Tecan. The mini-protein tetra system from Bio-Rad was used for the electrophoresis of SDS–PAGE gels.

2.2. Synthesis and characterization of magnetic particles

2.2.1. Synthesis of iron oxide magnetic particles

The synthesis of iron oxide MPs followed the co-precipitation method, using ferric and ferrous sulfate. For synthesis, 2.36 g of \( \text{FeSO}_4 \cdot 7\text{H}_2\text{O} \) and 6.79 g of \( \text{Fe}_2(\text{SO}_4)\cdot5\text{H}_2\text{O} \) were dissolved in 150 ml of deionized water, preheated to 80 °C in an inert atmosphere. When the temperature reached 80 °C, 50 ml of ammonium hydroxide of 25% (v/v) concentration was added and vigorously stirred for 30 min at 1200 r.p.m. After 30 min, the resulting MPs were collected using a permanent magnet and thoroughly washed with deionized water.

2.2.2. Silica and tetraethoxy silane coating of iron oxide magnetic particles

The silica coating was carried out according to method described by Lin et al. [18] with minor modifications. Briefly, 120 ml of aq. solution containing 3.4 g of \( \text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O} \) and 0.4 g of \( \text{NaOH} \) was introduced into the resultant particles after removal of deionized water from them using a permanent magnet. Before addition, the mixture was sonicated for 5 min to obtain a homogeneous solution of sodium silicate. The coating process was carried out using mechanical stirring for 30 min at slow agitation, which will only allow gentle mixing of solution and particles. This step was followed by the dropwise addition of 2 ml of 1 M HCl. Further, the particle mixture was added to 200 μl of TEOS dissolved in 1 ml of ethanol (use excess TEOS in order to get better coating); the addition was carried out with gentle mechanical stirring for 1 h. The entire process was carried out at room temperature. At the end of the coating process, the particles so prepared were washed with deionized water.

The MPs so prepared were then diluted to a concentration of 10 mg ml\(^{-1}\) and then coated with dextran. Aqueous solutions of dextran (2 and 4 g of dextran separately dissolved in 50 ml of distilled water) were prepared and added to 50 ml of MPs to yield MP-DX-1 and MP-DX-2, respectively. The mixtures were stirred mechanically for 2 h at room temperature, after which particles were washed with water. The amount of dextran released during the washings was quantified by the anthrone method, following the procedure described in Santana et al. [16].

2.2.3. Amino phenyl boronic acid coating of dextran-coated magnetic particles

Three different methods were tested for the modification of MP-DX-2 with APBA. The first method consisted of redispersion of 50 ml of MP-DX-2 (10 mg ml\(^{-1}\) MP) in 50 ml of ethanol by ultrasonication, followed by addition of 1 ml of APTES and stirred mechanically for 1 h at room temperature. The resultant particles were washed with distilled water and then redispersed in 25 ml of PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) containing 10 ml of 25% glutaraldehyde, and left under mechanical stirring for 30 min. After that, particles were washed several times with distilled water and then dispersed in 50 ml of PBS solution containing 0.5 g of APBA (previously neutralized with base) and 0.2 g of sodium cyanoborohydride. The mixture was then stirred mechanically for 3 h at room temperature. The obtained particles (MP-BA-I) were washed five times with distilled water and used for purification study.

The second method involved the reaction of MP-DX-2 with APS to form a complex organic net to hold the APBA molecule on the surface of MP yielding MP-BA-2. Briefly, 50 ml of MP-DX-2 (10 mg ml\(^{-1}\)) reacted with 4 g of APS and 0.4 g of neutralized APBA, for 2 h at room temperature with mechanical stirring, after which particles were washed with distilled water.

The third method included the reaction of MP-DX-2 with GLYMO to introduce active epoxide groups for further reaction with the free amine of APBA. A 50 ml bolus of MP-DX-2 with a concentration of 10 mg ml\(^{-1}\) was reacted with GLYMO for 3 h, at room temperature under mechanical stirring, after which 0.4 g of APBA (previously neutralized with NaOH) was added to the mixture and left reacting overnight. After completion of the reaction, the final MP-BA-3 particles were washed five times with distilled water and used for further study.

The prepared MPs were characterized by using a vibrating sample magnetometer (VSM; DSM 880) at the INESC-MN facilities (Lisbon, Portugal). The samples were prepared in milli-Q water with a concentration of 6.1 mg ml\(^{-1}\) and 30 μl of each sample were used in a vertical quartz rod. Transmission electron microscopy (TEM) was used for the characterization of particle morphology and estimation of the size of the magnetic core.

2. Material and methods

2.2. Synthesis and characterization of magnetic particles

2.2.1. Synthesis of iron oxide magnetic particles

The synthesis of iron oxide MPs followed the co-precipitation method, using ferric and ferrous sulfate. For synthesis, 2.36 g of \( \text{FeSO}_4 \cdot 7\text{H}_2\text{O} \) and 6.79 g of \( \text{Fe}_2(\text{SO}_4)\cdot5\text{H}_2\text{O} \) were dissolved in 150 ml of deionized water, preheated to 80 °C in an inert atmosphere. When the temperature reached 80 °C, 50 ml of ammonium hydroxide of 25% (v/v) concentration was added and vigorously stirred for 30 min at 1200 r.p.m. After 30 min, the resulting MPs were collected using a permanent magnet and thoroughly washed with deionized water.
The dried particle samples were prepared by evaporating dilute suspensions on a carbon-coated film and TEM performed in an analytical TEM Hitachi 8100 with a Ronset standard EDS detector and digital image acquisition. For all supports, the physical properties (hydrodynamic diameters and zeta potential) were determined by dynamic light scattering (DLS), using a Zetasizer Nano ZS from Malvern. For these analyses, samples with a final concentration of 0.05 mg ml$^{-1}$ in milli-Q water were prepared.

2.3. Binding pure protein solutions to magnetic particles

A 0.5 ml volume of MP-BA-3 with a concentration of 10 mg ml$^{-1}$ was washed sequentially with 0.5 ml of regeneration buffer (0.1 M NaOH in 30% (v/v) isopropanol) and milli-Q water and with elution buffer (1 M Tris–HCl of pH 8.5) followed by washing five times with binding buffer (20 mM HEPES, pH 8.5). After that, the supernatant was removed and resuspended in 0.5 ml of protein sample (BSA or IgG of concentration 1 mg ml$^{-1}$ in binding buffer) and incubated for 15 min at room temperature and an agitation speed of 300 r.p.m. The supernatant was recovered, and the matrix sites available to the partitioning solute (which can be fitted with Scatchard plot and are represented by $q = \frac{Q_{\text{max}} \cdot C_{\text{eq}}}{K_d + C_{\text{eq}}}$, where $q$ is the amount of bound protein in equilibrium per volume of solid support, $C_{\text{eq}}$ is the concentration of bound protein in equilibrium, and corresponds to the maximum concentration of the matrix sites available to the partitioning solute (which can also be defined as the binding capacity of the adsorbent) and the dissociation constant.

2.4. Characterization of boronic-acid-coated particles

2.4.1. Binding of alizarin red to magnetic particles

The AR test was carried out using a 5 mM stock solution of AR. A 250 µl sample of MPs (10 mg ml$^{-1}$) was incubated with the 250 µl AR solution for 15 min. After incubation, particles were first washed twice with 250 µl of binding buffer followed by washing five times using 250 µl of elution buffer. The entire analysis was carried out using two types of buffer conditions, i.e. phosphate–glycine and HEPES–Tris, HCl. In the case of phosphate–glycine, the adsorption and elution condition consisted of phosphate buffer at pH 7.4 and glycine buffer of pH 11, whereas HEPES–Tris, HCl consisted of HEPES buffer at pH 8.5 and Tris–HCl at pH 8.5. The major interest in using the AR test was to detect florescence to ascertain the presence of APBA coating on the surface of MPs.

2.4.2. Partition equilibrium studies

Partition equilibrium experiments were performed with solutions of human IgG (0–25 mg ml$^{-1}$; 250 µl) in binding buffer (20 mM HEPES) and 250 µl at 10 mg ml$^{-1}$ of MP-BA-3. For control, MP-DX-2 particles were used in order to study relative adsorption. The samples were incubated for 12 h at room temperature, after which the supernatants were collected and the amount of free protein quantified by the BCA protein assay. Experimental data were fitted with Scatchard plot and are represented by $q = \frac{Q_{\text{max}} \cdot C_{\text{eq}}}{K_d + C_{\text{eq}}}$.

2.4.3. Particle reuse study

After studying the supports for hlgG purification, the best supports, MP-BA-3, were tested for reuse applicability study with the purpose of testing the ability to reuse the support in purification processes. The APBA-functionalized products (MP-BA-3, 500 µl with 10 mg ml$^{-1}$) were tested with 500 µl of each of the pure hlgG solution, by incubating for 15 min at room temperature. The liquid in which particles were suspended was removed by magnetic separation and then washed two times with 500 µl of binding buffer (20 mM HEPES, pH 8.5). After washing, MPs were eluted five times using 500 µl of elution buffer (1 M Tris–HCl, pH 8.5). All eluted samples were analysed using the BCA method for hlgG quantification. In the next step, eluted MPs were regenerated using regeneration buffer (0.1 M NaOH in 30% (v/v) isopropanol), and again, the initial process step was repeated. In this way, five cycles were carried out in order to know reuse suitability.

2.4.4. Studies of incubation time

The time required for hlgG to adsorb on the surface of MPs having functionalized APBA molecules on the surface was studied in order to finalize the exact incubation time needed for optimum separation. In this study, five different test samples of MP-BA-3 (10 mg ml$^{-1}$ concentration) of 500 µl volume were incubated at room temperature with 500 µl of hlgG solution (1 mg ml$^{-1}$) for different time intervals: 5, 8, 10, 12 and 15 min. Incubation was followed by washing the particles two times with 500 µl of binding buffer (20 mM HEPES, pH 8.5) and eluting five times using elution buffer (1 M Tris–HCl of pH 8.5), in order to know the maximum amount protein that could be eluted. All washes were collected and quantified by the BCA method.

2.5. Purification of antibodies from unpurified MAb solutions

After studying the magnetic supports with pure solutions of hlgG, the selected magnetic material (MP-BA-3) and the control particles (MP-DX2) were tested with CHO cell culture supernatants. A 500 µl bolus of MP-DX-2 and MP-BA-3 (10 mg ml$^{-1}$) was incubated with 500 µl of the crude extract, respectively, without any pre-treatment, for 15 min at room temperature. Supernatants were collected, and the particles were washed five times with 500 µl of binding buffer (20 mM HEPES, pH 8.5). After washing, MPs were eluted using elution buffer (1 M Tris–HCl of pH 8.5), in order to study the best elution conditions. All collected samples were quantified by affinity chromatography using the Akta purifier system from GE Healthcare (Uppsala, Sweden), using the porous protein A affinity column from Applied Biosystems (Foster City, CA, USA). The BCA method and gel electrophoresis (12.5% acrylamide/bisacrylamide) under denaturing conditions by SDS–PAGE was also used for further analysis. The respective gels were prepared according to a standardized protocol. The low molecular weight marker and the samples for running gels were prepared by adding 2.5 and 10 µl of each sample, respectively, and 5 µl of sample buffer and boiled for 2 min immediately before applying to the gel. The gel was run for 80 min at 150 V and 250 mA by adding an electrophoresis Tris–glycine buffer (SDS–PAGE). For detection of the protein bands, the gel was stained using the silver stain Bio-Rad kit.

3. Results and discussion

3.1. Selection of the magnetic materials for antibody adsorption

An ideal support for affinity-based separations must be inert but still present chemical groups for further handling of specific receptors. The non-specific binding of bare iron oxide is a serious concern for selective adsorption and desorption in protein magnetic fishing. The crucial challenge of non-specific binding can be overcome by coating iron oxide
particles with biopolymers. We have previously reported the use of dextran as a preferential biopolymer to introduce an inert and functional layer onto MPs [16]. Dextran, when compared with other negatively charged biopolymers previously tested (e.g. gum Arabic and CM-dextran), presents an increased inertness probably owing to its neutral nature and the avoidance of undesired electrostatic interactions [19].

In this work, the produced MPs were firstly tested for binding to pure solutions of human IgG (hIgG, the target biomolecule) and BSA (a model non-glycosylated contaminant protein; table 1 and figure 2a). Bare MPs and TEOS-coated MPs show non-specificity as they bound between 45 and 80 mg protein g \(^{-1}\) of magnetic support. To decrease this undesired reactivity, particles were coated with a low and a

Table 1. Quantity of BSA and IgG bound and eluted from magnetic supports under different modification stages. Low concentration dextran coating is represented by DX-1 whereas high concentration dextran coating is represented by DX-2. (Binding buffer-20 mM HEPES of pH 8.5; elution buffer: 1 M Tris – HCl of pH 8.5; \(n = 4\).)

<table>
<thead>
<tr>
<th></th>
<th>IgG bound (mg g (^{-1}))</th>
<th>IgG eluted (mg g (^{-1}))</th>
<th>BSA bound (mg g (^{-1}))</th>
<th>BSA eluted (mg g (^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP-TEOS</td>
<td>53 ± 4</td>
<td>12 ± 3</td>
<td>70 ± 6</td>
<td>23 ± 5</td>
</tr>
<tr>
<td>MP-DX-1</td>
<td>38 ± 5</td>
<td>14 ± 4</td>
<td>8 ± 2</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>MP-DX-2</td>
<td>3 ± 4</td>
<td>1 ± 4</td>
<td>2 ± 4</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>MP-BA-3</td>
<td>173 ± 2</td>
<td>166 ± 3</td>
<td>10 ± 2</td>
<td>4 ± 2</td>
</tr>
</tbody>
</table>

Figure 1. (a) Modification of dextran-coated MP via the GLYMO route. GLYMO coating leaves a reactive epoxide moiety which is subsequently reacted using 3-aminophenyl boronic acid (3-APB) to give the APBA-functionalized product. (b) Alkyl or aryl boronic acids react with cis-diol hydroxyls on 6-membered pyranose and five-membered furanose constituting carbohydrates under alkaline conditions to give boronate esters. Note the change from trigonal planar to tetrahedral geometry upon esterification, leaving a negative charge on the boron atom.
high concentration of dextran. It has been suggested that the dominant mechanism of the interaction between dextran and MPs is the formation of collective hydrogen bonding between dextran hydroxyl groups and the iron oxide particle surface. Coating of bare MPs with silicon dioxide and tetraethoxysilane creates a spongy adsorptive surface with high adsorption capacity to polymeric substances. The silica-coated structure is a network of silicon atoms connected by oxygen atoms and saturated with hydroxyl groups. The external coating of silica easily adsorbs an efficient amount of dextran solution on its surface. The maximum quantities of dextran coated on the surface of TEOS-coated MPs were 1.1 (mg g$^{-1}$ of MP) for low and 1.4 (mg g$^{-1}$ of MP) for the higher concentration. It was observed that after biopolymer coating, non-specific binding of BSA was reduced to 8 mg g$^{-1}$ of support for the low concentration of dextran coating and almost negligible for the high concentration (table 1 and figure 2a). The major reason behind a reduction in non-specific binding is the creation of a carbohydrate-based hydrophilic and uncharged layer around the iron oxide particles which imparts greater inertness. In addition, dextran-coated MPs become more stable. The iron oxide particles coated with the higher amount of dextran (MP-DX-2) were selected to proceed further with ligand attachment.

Three different approaches were investigated for the attachment of boronic acid at the surface of the MP-DX-2 particles. The different magnetic supports produced were tested for binding to hIgG and BSA, showing that APBA attachment through the GLYMO spacer yielded the best results (table 1 and figure 2a), as the affinity and specificity towards IgG was higher. Therefore, MP-BA-3 particles were selected for further studies and characterization.

When comparing MP-BA-3 particles with other MPs modified with synthetic ligands for antibody purification, these present competitive potential. The experimental adsorption values of ligand 22/8 modified MP with dextran and gum arabic coating was found to be 130 and 110 mg hIgG adsorbed g$^{-1}$ of MP, respectively [16]. The commercially available APBA-modified MP shows experimental adsorption of 109 mg hIgG adsorbed g$^{-1}$ MP [17], whereas MP-BA-3 particles adsorb 173 mg hIgG g$^{-1}$ MP.

The main differences between the commercial particles SiMAG–boronic acid and the particles described in this work are (i) commercial particles have an hydrodynamic diameter of 1 μm and ours possess particles with heterogeneous sizes between 200 and 2700 nm (figure 4a); (ii) the core of the commercial particles is maghemite, whereas ours is magnetite; (iii) the primary coating of our particles...
is porous silicate with dextran which differs from the commercial ones—SiMAG beads have an inert and stable non-porous solid-phase silica matrix; (iv) the method of APBA attachment onto commercial particles is not known, and therefore it is not possible to compare with our data. As shown in figure 1, the different types of coating and functionalization methods and particles produced from them such as MP-BA-1, MP-BA-2 and MP-BA-3 have wide variations in hIgG and BSA binding. Therefore, it is likely that the differences between the particles produced in this work and the commercial beads fully justify the differences observed. Dextran reduces non-specific binding and makes the magnetic support inert, as shown in this work and previously in [16], so that the actual binding was mainly through APBA coating on the surface.

The first characterization performed concerned investigation of the correct structural display of boronic acid at the surface of MPs. AR is known to be a reporter to study carbohydrate–boronic acid interactions owing to its change in fluorescence intensity upon binding to a boronic acid [20]. MP-BA-3 particles were tested for binding and eluting AR using two types of buffer conditions, previously shown to adsorb and elute IgG from commercial boronic acid particles [17]. From the results shown in figure 2, it is observed that for binding with phosphate buffer pH 7.4 and elution with glycine buffer pH 11, MP-BA-3 particles adsorbed approximately 40% AR and eluted approximately 30%. When binding and elution occur at pH 8.5 with HEPES/Tris–HCl buffers, MP-BA-3 particles adsorbed approximately 55% AR and eluted approximately 50%. For both buffer conditions tested, the control particles (MP-DX-2) bound a negligible amount of AR.

Table 2. Comparison of affinity constants and theoretical maximum binding capacities of human IgG binding by several magnetic supports.

<table>
<thead>
<tr>
<th>support type</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$Q_{\text{max}}$ (mg hIgG adsorbed g$^{-1}$ support)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP-BA-3</td>
<td>$4.9 \times 10^5$</td>
<td>492</td>
</tr>
<tr>
<td>SiMAG—boronic acid$^a$</td>
<td>$2.9 \times 10^4$</td>
<td></td>
</tr>
<tr>
<td>commercial protein A on MP$^b$</td>
<td>$3.3 \times 10^5$</td>
<td>109</td>
</tr>
<tr>
<td>ligand 22/8 on MP_DX$^b$</td>
<td>$7.7 \times 10^5$</td>
<td>568</td>
</tr>
<tr>
<td>protein A on agarose$^b$</td>
<td>$3.7 \times 10^5$</td>
<td>17</td>
</tr>
</tbody>
</table>

$^a$Values estimated at pH 8.5 while fitting experimental data with the Freundlich isotherm [14].

$^b$From Xu et al. [13].

Figure 3. Binding of human IgG at the surface of MP-Dex modified with APBA by GLYMO method MP-BA-3 (a,b) and MP-DX-2 control (c,d). Representation of $q$ (the amount of bound hIgG in equilibrium per mass of solid support) as function of $C_{eq}$ (the concentration of hIgG in equilibrium). Experimental data were fitted with the expression $q = (Q_{\text{max}} \times C_{eq})/(K_d + C_{eq})$ using Scatchard plot, where $Q_{\text{max}}$ corresponds to the maximum concentration of the matrix sites available to the partitioning solute (which can also be defined as the binding capacity of the adsorbent), and $K_d$ is the dissociation constant.
indicating a correct display of the boronic acid moiety at the surface of the particles.

The selected MP-BA-3 particles were further used to study the binding kinetics and the reuse potential. As shown in figure 2d, the minimum time required for the maximum adsorption of IgG was estimated at 12 min, which was in accordance with the previous results obtained with commercial boronic acid particles [17], corroborating the fast kinetics of the interaction between APBA particles and the glycosylated IgG. Regarding the studies to ascertain the regeneration and reuse capability of the MP-BA-3 particles, it was observed that until the fifth stage of recycling, particles retained about 75–80% of protein binding capacity (when considering initial protein binding capacity as 100%; figure 2c).

Finally, the static binding capacity of MP-BA-3 particles was estimated by partition equilibrium experiments with pure solutions of hIgG. The adsorption isotherm of hIgG onto MP-BA-3 could be fitted to a Langmuir profile yielding an affinity constant of $4.9 \times 10^4$ M$^{-1}$ ($K_a$) and a theoretical maximum capacity of 492 mg hIgG adsorbed g$^{-1}$ MP ($Q_{\text{max}}$) with a correlation factor of 0.93. For the control (MP-DX-2), which binds a negligible amount of hIgG, the determined theoretical
maximum capacity was 3.1 mg hIgG adsorbed g⁻¹ MP (Q_max). The values of K_a determined for MP-BA-3 were lower than those estimated for the commercial boronic acid particles under the same experimental conditions, where the adsorption constant K was estimated as 1.5 × 10⁸ M⁻¹ when fitting a Freundlich isotherm to the experimental dataset (figure 3).

In terms of Q_max value (table 2), the MP_Ga and MP_Dx modified with ligand 22/8 has a theoretical maximum capacity of 344 mg and 568 mg hIgG adsorbed g⁻¹ MP [16], for ligand 22/8, on a cellulose membrane, the values were in the range of 630 mg hIgG adsorbed g⁻¹ MP; the commercially available APBA MPs shows adsorption values 378 of mg hIgG adsorbed g⁻¹ MP, whereas MPs as per this study shows Q_max 492 mg hIgG adsorbed g⁻¹ MP.

3.2. Characterization of the magnetic materials for antibody adsorption

The selected magnetic support for IgG purification (MP-BA-3) and the control unmodified particles (MP-Bare, MP-DX-2) were characterized by DLS to infer on the hydrodynamic diameter. As shown in figure 4a, the average hydrodynamic diameter increased from 255–270 nm to 700–720 nm after dextran and APBA coating. In the latter, the MP population is more heterogeneous in composition. The increase of MP size upon biopolymeric coating has already been observed in other systems where gum arabic and dextran were employed [16].

In order to better control particle aggregation, synthesis and modification procedures could have been carried out with high-speed agitation and ultrasound treatment. The separation of particles by size would be possible by application of magnets with increasing magnetic strengths or by high gradient magnetic separation. The particles used for bioseparation processes must balance the hydrodynamic diameter, related with the surface area and the ease of separation. Very small magnetic nanoparticles (hydrodynamic diameters less than 200 nm) are very difficult to separate with commonly available permanent magnets and also take a long time to separate (which can be deleterious to the protein particularly during elution steps); if using an electromagnet on high-gradient-magnetic-separation, small particles require high magnetic fields for separation (which contributes on its own for particle aggregation) and require a very tight control on the fluxes applied.

Regarding the variation of zeta potential with different pH values, results in figure 4b indicate that control particles (MP-DX-2) have a zeta potential in the range of −5 mV at pH 3 varying gradually with the pH1 until reaching −9 mV at pH 11. In the case of MP-BA-3, the zeta potential variation with pH1 is very similar to the behaviour observed for the control particles between pH 3 and 8 (figure 4b), being −6.3 mV at pH 3. However, from pH 8 onwards, and particularly when shifting from pH 9 to 10, there is a considerable drop in the zeta potential values (from −14 to −23 mV). These results are associated with the transition from trigonal to tetrahedral structure at the pH of boronic acids, estimated as 8.4 and 9.2 for free and immobilized phenyl boronic acids, respectively.

The characterization of particles by TEM confirmed the existence of spherical magnetic cores with an average diameter of 14 nm. Some heterogeneous population is also observed, because the size distribution is between 6 and 20 nm (figure 4c–f). With VSM analysis, it was possible to investigate the magnetic properties of the supports prepared. The curves shown in figure 4g represent a typical no hysteresis curve with reversibility and symmetry, characteristic of the superparamagnetic behaviour of the particles synthesized. In terms of saturation magnetization, the values obtained were 37 emu g⁻¹ for MP-DX-2, and 34 emu g⁻¹ for MP-BA-2. The saturation magnetization value obtained for the MPs is consistent with the values referenced in [17] for commercial dibenzofuran boronic-acid-coated MPs in the range of 35 emu g⁻¹. The XRD spectra represented in figure 4h show five characteristic peaks for Fe₃O₄ marked by their indices (2 2 0), (3 1 1), (4 0 0), (5 1 1), (4 4 0)). These peaks are consistent with the International Centre for Diffraction Data (ICDD 2007) and revealed that the resultant MPs were Fe₃O₄ (figure 5).

3.3. Tests with crude samples

After preliminary studies with pure solutions of hIgG and BSA, the MP-BA-3 supports were tested with a CHO cell culture supernatant in order to verify the applicability of the magnetic supports to capture antibodies from complex mixtures. Under reducing conditions, antibodies dissociate into several structures of molecular weight 100, 50 and 25 kDa. After confirmation by SDS–PAGE of the binding and elution capacity of the support on the crude extract used, the samples collected were quantified by the BCA method in order to determine the amount of protein bound to and eluted from the support and hence the purity of this recovery. From the
BCA results, it was possible to predict that approximately 91% of total protein bound to the support. Using HPLC for quantification of IgG fractions, it was observed that about 98% of IgG from crude sample bound to the MPs, and it is possible to elute 95% of pure IgG at pH 8.5. The quantity of IgG eluted in the five fractions was 21%, 19%, 20%, 17% and 18%.

4. Conclusion

Synthesis and study of MPs coated with APBA and modified using GLYMO led to particles exhibiting promising characteristics for application in bioseparation processes. Human IgG purification will be the most suitable and desirable option from a separation point of view. Synthesis and modification of magnetic support using the methods described in this study showed encouraging results in terms of production and utilization efficiency. The support showed superior performance under elution conditions despite the absorption of some (albeit a relatively small) quantity of undesired proteins. Nevertheless, the overall performance for human IgG separation was better than expected. One more advantage of this support is that it showed low non-specific adsorption in the presence of BSA and no major loss of the capacity of the support when reused up to five times. From these studies, it is possible to conclude that approximately 98% of the desired protein from crude extract was bound to the support, and at pH 8.5 using Tris–HCl elution conditions it was possible to elute approximately 95% desired protein based on adsorption.

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References