An extracellular polymer at the interface of magnetic bioseparations

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Abstract

FucoPol, a fucose-containing extracellular polysaccharide (EPS) produced by bacterium Enterobacter A47 using glycerol as the carbon source, was employed as a coating material for magnetic particles (MPs), which were subsequently functionalized with an artificial ligand for the capture of antibodies. The performance of the modified MPs (MP–EPS-22/8) for antibody purification was investigated using direct magnetic separation alone or combined with an aqueous two-phase system (ATPS) composed of polyethylene glycol (PEG) and dextran. In direct magnetic capturing, and using pure protein solutions of human immunoglobulin G (hIgG) and bovine serum albumin (BSA), MP–EPS-22/8 bound 120 mg hIgG g\(^{-1}\) MPs, whereas with BSA only 10 \(\pm\) 2 mg BSA g\(^{-1}\) MPs was achieved. The hybrid process combining both the ATPS and magnetic capturing leads to a good performance for partitioning of hIgG in the desired phase as well as recovery by the magnetic separator. The MPs were able to bind 145 mg of hIgG g\(^{-1}\) of particles which is quite high when compared with direct magnetic separation. The theoretical maximum capacity was calculated to be 410 \(\pm\) 15 mg hlgG adsorbed g\(^{-1}\) MPs with a binding affinity constant of 4.3 \(\times\) 10\(^{-4}\) M\(^{-1}\). In multiple extraction steps, the MPs bound 92\% of loaded hIgG with a final purity level of 98.5\%. The MPs could easily be regenerated, recycled and re-used for five cycles with only minor loss of capacity. FucoPol coating allowed both electrostatic and hydrophobic interactions with the antibody contributing to enhance the specificity for the targeted products.

1. Introduction

The need for biological molecules such as proteins, gene or viral vectors and cells in highly purified forms is usually a challenging and costly task [1]. Antibodies are an extremely important class of biopharmaceuticals with the market value now reaching US$66.5 billion [2]. In this respect, chromatography is the most well-established separation method, in both laboratory and industrial settings. However, the limitations of the chromatographic methods to accompany increasing production titres, the flexibility in production and the associated high costs are driving the search and re-evaluation of non-chromatographic technologies. Within these, aqueous two-phase systems (ATPSs) and magnetic separation appear as potential candidates to be employed in combination or as a replacement for the chromatographic steps [3]. Very recently, our groups presented a novel methodology which combined aqueous two-phase extraction (ATPE) with magnetic capture, known as a hybrid process, which gave high antibody recovery yields (92\%) with high purity (98\%) [4].

In order to employ magnetic particles (MPs) in bioseparation processes, the magnetic core needs to be coated with either biological or synthetic polymers. The main role of the MP coating is the protection of the core structure with an encapsulation layer in order to isolate the core against harsh conditions. The polymer coating also improves the biocompatibility of the particles and increases the functionalization for further modifications [5–7]. The coatings most commonly used include: organic materials, such as surfactants [8,9] and polymers, or inorganic materials, such as silica [10], carbon [11,12] and precious metals [13,14]. In recent
years, the coating of MPs with polymers, particularly biopolymers such as polysaccharides [15], attracted the attention of researchers as they are known to increase biocompatibility [16], chemical functionality and colloidal stability of nanomaterials [17]. In addition, biopolymers are renewable, non-toxic and biodegradable materials, which makes them an environmental and sustainable choice [18]. The combination of natural polymers with superparamagnetic particles results in the generation of stable materials with low unspecific binding and available chemical groups for further ligand binding as well as attachment of enzymes, which plays an important role in biocatalysis [19] and bioseparation processes [20].

The polysaccharides produced by microorganisms can be divided into intracellular, structural and extracellular polysaccharides or exopolysaccharides (EPSs). Over recent decades, several EPSs have been reported, and their composition, structure, biosynthesis and functional properties have been extensively studied [21]. EPSs are synthesized intracellularly by cells and exported to the extracellular environment as macromolecules, which makes their extraction process easier than for other natural polysaccharides [22]. EPSs are characterized by interesting functional properties that include their rheology, their ability to modify an aqueous medium, their emulsion-forming and stabilizing capacity, their flocculating activity and their filmogenic capacity [23].

EPSs are mainly composed of neutral sugar monomers, but acidic or amino sugars are also common constituents [24]. Although glucose and galactose are the most common sugar residues in EPS structures, some polysaccharides have an increased value owing to their content in certain sugars that occur rarely in nature, such as fucose. Fucose-containing EPSs have an increased market value, because fucose is one of the rare sugars, is difficult to obtain, and has many different applications that include cosmetics and pharmaceuticals. Fucose-containing EPSs have been reported to be produced by several bacterial genera, including Klebsiella, Clostridium, Escherichia and Enterobacter [25]. In this work, FucoPol, a fucose-containing EPS polymer synthesized by the bacterium Enterobacter A47 (DSM 23139) [22] and having unique characteristics [21,24], was used as a coating material for MPs. FucoPol-coated particles were then functionalized with an artificial ligand to capture antibodies through magnetic capturing and a hybrid process combining both ATPS and magnetic capturing (table 1).

2. Material and methods

2.1. Chemicals

Cyanuric chloride, tris(hydroxymethyl)aminomethane, (3-aminopropyl)triethoxysilane (APTES), 3-hydroxyaniline, ferric sulfatehydrate, ferrous sulphate heptahydrate, HEPES, anthrone, sulfuric acid, absolute ethanol, hydrochloric acid, sodium chloride, sodium-di-hydrogen phosphate monohydrate, di-sodium hydrogen phosphate di-hydrate and sodium hydride were purchased from Sigma-Aldrich. Polyclonal human immunoglobulin G (hiG) for therapeutic administration (product name: Gamma norm) was purchased from Octapharma (Buchs, Switzerland), as a 165 g l⁻¹ solution containing 95% IgG. 4-Amino-1-naphthol hydrochloride and poly(ethylene glycol) with molecular weights 3350 and 8000 Da were purchased from Sigma (St. Louis, MO). Dextran with an average molecular weight of 500 000 Da was purchased from Fluka (Buchs, Switzerland). Glycine was purchased from Acros. Nihydrin and ammonium hydroxide were purchased from Fluka.

2.2. Methods

2.2.1. Biopolymer production

The microorganism used in this study was the bacterium Enterobacter A47 (DSM 23139). The culture was preserved in glycerol (20%, v/v) as a cryoprotectant agent, at −80 °C. Reactivation from the stock cultures was performed in Luria broth (LB) medium, which was also used to prepare inocula for the assays. In the bioreactor experiments, Enterobacter A47 was grown on a slightly modified medium E⁺ (pH 7.0), as previously described [25]. Medium E⁺ was supplemented with glycerol (approx. 40 g l⁻¹).

Inocula for the assays were prepared by inoculating 20 ml of LB medium grown cells into 200 ml fresh LB medium and incubating the culture in an orbital shaker for 48 h (at 30 °C, and 150 r.p.m.). Afterwards, the culture was transferred again (80 ml) to fresh medium E⁺ (800 ml) and further incubated for 72 h. The 5 l bioreactor (BioStat B-plus, Sartorius) containing 4 l of medium E⁺, supplemented with glycerol (at a concentration of approx. 25 g l⁻¹), was inoculated with the culture (800 ml). The bioreactor was operated as previously described [25]. Temperature and pH were controlled at 30 ± 0.1 °C and 7.00 ± 0.05, respectively. The aeration rate (0.125vvm, volume of air per volume of reactor per minute) was kept constant throughout the cultivation, and the dissolved oxygen (DO) concentration was controlled at 10% air saturation by the automatic variation of the stirring speed between 300 and 800 r.p.m. The bioreactor cell growth was limited by nitrogen exhaustion, followed by supplying the bioreactor with cultivation medium E⁺, with a glycerol concentration of 200 g l⁻¹, at a constant rate of 10 ml h⁻¹. Culture broth samples were collected from the bioreactor over time in order to evaluate the bacteria’s growth, culture broth viscosity and quantification of biomass, nutrient and polymer production.

For recovery of the EPS from the broth, it was diluted with deionized water (1:5, v/v) for viscosity reduction and centrifuged (8000 r.p.m., 1 h). The cell-free supernatant was subjected to thermal treatment (70 °C, 1 h) to inactivate bacterial enzymes that might cause polymer degradation during the subsequent purifications. Afterwards, denatured proteins and any remaining bacterial cells were removed by centrifugation (8000 r.p.m., 1 h), and the cell-treated supernatant was purified by ultra/diafiltration, using a hollow fibre module with a 500 kDa molecular weight cut-off (MWCO) membrane, and freeze-dried. Further details on EPS production and extraction can be found in the electronic supplementary material.

2.2.2. Extracellular polysaccharide coating and functionalization of magnetic particles

Basic magnetic core synthesis of MPs was carried out using ferric and ferrous chloride solutions as previously described [3]. The coating process was then carried out by mechanically stirring the tetraethoxysilane-coated MPs with a solution of EPS polymer. After mechanical stirring, the particles were washed five times with distilled water and aminated using (3-aminopropyl)triethoxysilane (APTES). Amination was followed by resuspension of particles in a 50% (v/v) acetic acid/water mixture and reaction with five molar equivalents of cyanuric chloride based on the amount of amines available on the support. The first nucleophilic substitution reaction was carried out using 3-hydroxyaniline in water, by adding two equivalents relative to the amount of amines on the supports. The time span of reaction was 24 h with stirring at 30 °C. After the reaction, the particles were washed five times with water. The second nucleophilic substitution was carried out using five molar equivalents of 4-amino-1-naphthol-hydrochloride, dissolved in 50% (v/v) dimethylformamide (DMF)/water. The reaction was carried out for 48 h with stirring at 90 °C. The particles were then washed once with 50% (v/v) DMF/water and five more times with water. Finally, the particles
Table 1. Comparison of the hybrid process (magnetic capturing plus ATPS) and direct magnetic capturing.

<table>
<thead>
<tr>
<th>Process</th>
<th>Yield (%)</th>
<th>Purity (%)</th>
<th>Amount of MPs needed per ml of crude</th>
<th>Time required for phase separation in ATPS (min)</th>
<th>Time needed for incubation (min)</th>
<th>Ligand attached to MPs</th>
<th>Mg of salt per g ATPS</th>
<th>Eluent buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct magnetic capturing from [4]</td>
<td>67</td>
<td>74</td>
<td>10 mg</td>
<td>n.a.</td>
<td>15</td>
<td>boronic acid</td>
<td>n.a.</td>
<td>Tris – HCl (pH 8.5)</td>
</tr>
<tr>
<td>Direct magnetic capturing (after three cycles) as per article</td>
<td>cycle 1</td>
<td>38</td>
<td>90</td>
<td>20 mg</td>
<td>n.a.</td>
<td>ligand-22/8</td>
<td>n.a.</td>
<td>glycine – NaOH (pH 11)</td>
</tr>
<tr>
<td></td>
<td>cycle 2</td>
<td>13</td>
<td>reuse</td>
<td>n.a.</td>
<td>15</td>
<td>ligand-22/8</td>
<td>n.a.</td>
<td>glycine – NaOH (pH 11)</td>
</tr>
<tr>
<td></td>
<td>cycle 3</td>
<td>7</td>
<td>reuse</td>
<td>n.a.</td>
<td>15</td>
<td>ligand-22/8</td>
<td>n.a.</td>
<td>glycine – NaOH (pH 11)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>58</td>
<td>87</td>
<td>20 mg</td>
<td>n.a.</td>
<td>ligand-22/8</td>
<td>n.a.</td>
<td>glycine – NaOH (pH 11)</td>
</tr>
<tr>
<td>ATPS system alone from [7]</td>
<td>23</td>
<td>39</td>
<td>n.a.</td>
<td>40</td>
<td>n.a.</td>
<td>n.a.</td>
<td>150 mg</td>
<td>n.a.</td>
</tr>
<tr>
<td>Hybrid process from [4]</td>
<td>92</td>
<td>98</td>
<td>5 mg</td>
<td>25</td>
<td>10–30</td>
<td>boronic acid</td>
<td>11.6 mg</td>
<td>Tris – HCl (pH 8.5)</td>
</tr>
<tr>
<td>Hybrid process (as per article)</td>
<td>cycle 1</td>
<td>62</td>
<td>98</td>
<td>10 mg</td>
<td>5–40</td>
<td>ligand-22/8</td>
<td>11.6 mg</td>
<td>glycine – NaOH (pH 11)</td>
</tr>
<tr>
<td></td>
<td>cycle 2</td>
<td>30</td>
<td>reuse</td>
<td>25</td>
<td>5–40</td>
<td>ligand-22/8</td>
<td>n.a.</td>
<td>glycine – NaOH (pH 11)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>92</td>
<td>98</td>
<td>10 mg</td>
<td>n.a.</td>
<td>ligand-22/8</td>
<td>11.6 mg</td>
<td>glycine – NaOH (pH 11)</td>
</tr>
<tr>
<td>Process in [13]</td>
<td>39</td>
<td>49</td>
<td>40 mg</td>
<td>n.a.</td>
<td>60</td>
<td>IgG</td>
<td>150 mg</td>
<td>3.5 M KSCN</td>
</tr>
</tbody>
</table>
modified with ligand 22/8 were resuspended in water. The magnetic particle modification is shown schematically in scheme 1.

2.2.3. Magnetic aqueous two-phase extraction
An ATPE system composed of 8% (w/w) polyethylene glycol (PEG) (3350 Da) and 5% (w/w) dextran (500,000 Da) was used to investigate the biopolymer-coated MPs' performance. For the preparation of the ATPS, stock solutions of PEG and dextran were weighed in 15 ml graduated glass tubes in order to obtain the final desired composition for a total system weight of 5 g. The synthesized MPs–EPS–22/8 were added at a final concentration of 0.02% (w/w). Pure hIgG extraction studies were performed by adding 1 ml of 1 g l$^{-1}$ hIgG stock solution. In supernatant hIgG extraction studies (1.35 g l$^{-1}$ hIgG), the supernatant loading of the systems ranged from 1 to 1.5 ml. Total final weight of the system (5 g) was balanced by adding Milli-Q water. Salt concentration was varied between 100 and 500 mM for all systems. After mixing all components thoroughly in a vortex shaker, the phases were allowed to separate for 2–4 h at room temperature. The MPs were then recovered by positioning the test tubes on a magnetic separator. Samples of each phase were also collected for further analysis. Adsorbed proteins were afterwards subjected to elution studies in order to assess the amount of hIgG adsorbed to the MPs. After removal of the two aqueous phases, five subsequent MP washes were made, the first one with Milli-Q water and the remaining ones with 50 mM phosphate buffer at pH 8. The elution was performed in five consecutive steps using 50 mM glycine–NaOH buffer at pH 11. The amount of IgG present in both ATPS phases and the amount recovered after elution from the MPs were determined by high-performance liquid chromatography (HPLC), as described in §2.2.6.

2.2.4. Direct magnetic capturing of antibodies
The functionalized MPs–EPS–22/8 were tested with pure solutions of hlgG and bovine serum albumin (BSA). The nanoparticle suspensions were firstly washed with 0.1 M NaOH prepared in 30% (v/v) isopropanol, followed by washing with deionized water. After washing, the MPs were equilibrated in the binding buffer (50 mM phosphate buffer at pH 8). After washing and equilibration, the MPs were suspended in 500 μl of a hIgG or BSA solution, both at 1 mg ml$^{-1}$ in binding buffer. The incubation was carried out for 15 min at room temperature with constant stirring. The supernatant was then collected, and the particles were washed five times with 50 μl of binding buffer. After washing the MPs, the elution of bound protein was carried out using 50 mM glycine–NaOH buffer at pH 11. All samples were analysed by bicinchoninic acid (BCA) assay with a microplate reader in order to quantify the amount of protein bound to and eluted from the MPs. In order to compare results, non-modified particles (MPs–EPS) and ligand-modified particles (MPs–22/8) were tested at the same time and under the same conditions as the magnetic supports (MPs–EPS–22/8).

2.2.5. Partition equilibrium studies
Partition equilibrium experiments were performed using 500 μl solutions containing hlgG at a concentration in the range of 0–25 mg ml$^{-1}$ in 50 mM phosphate buffer at pH 8. The concentration of the MPs was maintained at 10 mg ml$^{-1}$ throughout the experiment. As a control, MPs–EPS 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 was quantified by the BCA protein assay. Experimental data fitted a 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, \(Q_{\text{max}}\) 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.

2.2.6. Regeneration and reuse of MPs–EPS–22/8
MPs–EPS–22/8 were tested in a reuse applicability study with the purpose of testing the ability to reuse the support in

Scheme 1. Schematic of ligand 22/8 modification of magnetic particles.
consecutive purification processes. The 22/8-functionalized MPs (500 μl with 10 mg ml⁻¹) were tested with 500 μl of the pure hIgG solution, by incubating for 15 min at room temperature. Then, the adsorption supernatant was removed by magnetic separation and washed twice with 500 μl of binding buffer (50 mM phosphate at pH 8). After washing, bound hIgG MPs were eluted in five sequential steps using 500 μl of elution buffer (50 mM glycine–NaOH at pH 11). All eluted samples were analysed using the BCA method for hIgG quantification. In the next step, eluted MPs were regenerated using a regeneration buffer composed of 0.1 M NaOH in 30% (v/v) isopropanol, and again the initial process step was repeated. In total, five cycles were carried out in order to study the reusability of the MPs.

2.2.7. Studies of incubation time for direct as well as aqueous two-phase extraction-based separation processes

Incubation time plays an important role in the adsorption of antibodies on the surface of the MPs. Functionalized MPs–EPS-22/8 were studied in order to define the incubation time that provides the optimum separation ability. This study was carried out separately for direct magnetic separation and magnetically enhanced aqueous two-phase extraction (MATPE). For the direct magnetic separation study, five different test samples of MPs–EPS-22/8 of volume 500 μl were incubated at room temperature with 500 μl hIgG solution (1 mg ml⁻¹) for different time periods of 5, 8, 10, 12, 14, 16, 18 and 20 min, respectively. Incubation was followed by washing the particles twice with 500 μl binding buffer (50 mM phosphate at pH 8) and eluting the bound hIgG five times using 500 μl elution buffer (50 mM glycine–NaOH at pH 11). All washes were collected and quantified by the BCA method. For the MATPE study, five different ATPSs were prepared as described in §2.2.4. The incubation time was varied for each system and allowed for phase separation. After phase segregation, particles were separated and washed as described above for the direct magnetic separation study in order to recover hIgG for further quantification.

2.2.8. Crude hIgG extracts purification

The magnetic supports MP–EPS-22/8 and MP–EPS were tested with CHO cell culture supernatants in order to estimate the efficiency of MPs for crude purification. For comparison of the results, testing of crude extract was carried out using both techniques, i.e. direct magnetic separation as well as MATPE. The components of the crude mixture are usually a mixture of DMEM (Gibco) and ProCHO5 (Lonza, Basle, Switzerland; v/v: 1:4) or only ProCHO5. DMEM was supplemented with 1.5 g l⁻¹ NaHCO₃ (Sigma), 200 mM MTX (Sigma), 2 mg l⁻¹ recombinant human insulin (Sigma), 35 mg l⁻¹ l-proline (Sigma), 0.1% (v/v) of a trace element A and B (Cellgro, Mediatech) and 1% (v/v) antibiotics (0.025 μg ml⁻¹ penicillin and 0.025 μg ml⁻¹ streptomycin, both from Gibco). The serum used was ultra-low IgG FBS (Gibco) at concentrations between 0.015 and 1.0 g l⁻¹. The acid hydrolysates were also used for the identification of the constituent sugar monomers by liquid chromatography (HPLC), using a CarboPac PA10 column (Dionex), equipped with an amperometric detector. The analysis was performed at 30 °C, with NaOH (4 mM) as the eluent, at a flow rate of 0.9 ml min⁻¹. Fucose, glucose, galactose and glucuronic acid (Sigma) were used as standards at concentrations between 0.005 and 0.1 g l⁻¹. The acid hydrolysates were also used for the identification and quantification of acyl groups by HPLC with an Aminex HPX-87H column (Bio-Rad) coupled to a UV detector. The analysis was performed using sulfuric acid (0.01 N) as the eluent, at a flow rate of 0.6 ml min⁻¹ and a temperature of 50 °C. Acetate, pyruvate and succinate (Sigma) were used as standards at concentrations between 0.015 and 1.0 g l⁻¹. An Äkta purifier system from GE Healthcare (Uppsala, Sweden) was used for measurement of the IgG concentration in the top and bottom phases to carry out the analysis. Quantification was done by using a porous protein A affinity column from Applied Biosystems. The IgG concentration was determined from a calibration curve obtained using Gammanorm IgG as a standard.

The Bradford method was used to determine the total protein concentration using an A¨ kta purifier system from GE Healthcare (Uppsala, Sweden) and a porous protein A affinity column from Applied Biosystems (Foster City, CA).

2.2.9. Analytical techniques

The culture broth viscosity was measured, immediately after sample collection, using a viscometer (Brookfield VD-II). The samples recovered over time were centrifuged at 10,000 r.p.m. for 10 min, for cell separation. The cell-free supernatant was stored at −20 °C for posterior determination of glycerol and ammonium concentrations, and for the quantification of the EPS produced. The cell pellet was used for the gravimetric determination of the cells’ dry weight (CDW), after washing three times with deionized water (resuspension in water, centrifugation at 10,000 r.p.m., for 10 min, and, finally, resuspension in deionized water), filtration through 0.2 μm membranes, and filter drying at 100 °C until constant weight.

The glycerol concentration in the cell-free supernatant was determined by HPLC with an Aminex HPX-87H column (Bio-Rad), coupled to a refractometer. The analysis was performed at 50 °C, with sulfuric acid (H₂SO₄, 0.01 N) as the eluent, at a flow rate of 0.6 ml min⁻¹. The ammonium concentration was determined using a potentiometric sensor (Thermo Electron Corporation, Orion 9212).

The cell-free supernatant was dialysed with a 10,000 MWCO membrane (SunnySkin pleated dialysis tubing, Thermo Scientific), against deionized water (48 h, 4 °C) and freeze-dried for EPS quantification. The dialysis solution contained 6 ppm sodium azide to avoid biological degradation of the samples. Afterwards, the EPS was analysed for its composition in sugar and acyl groups. Briefly, EPS dried samples (approx. 3 mg) were dissolved in deionized water (5 ml) and hydrolysed with trifluoroacetic acid (TFA; 0.1 ml TFA 99%) at 120 °C for 2 h. The hydrolysate was used for the identification of the constituent sugar monomers by liquid chromatography (HPLC), using a CarboPac PA10 column (Dionex), equipped with an amperometric detector. The analysis was performed at 30 °C, with NaOH (4 mM) as the eluent, at a flow rate of 0.9 ml min⁻¹. Fucose, glucose, galactose and glucuronic acid (Sigma) were used as standards at concentrations between 0.005 and 0.1 g l⁻¹. The acid hydrolysates were also used for the identification and quantification of acyl groups by HPLC with an Aminex HPX-87H column (Bio-Rad) coupled to a UV detector. The analysis was performed using sulfuric acid (0.01 N) as the eluent, at a flow rate of 0.6 ml min⁻¹ and a temperature of 50 °C. Acetate, pyruvate and succinate (Sigma) were used as standards at concentrations between 0.015 and 1.0 g l⁻¹.

An Äkta purifier system from GE Healthcare (Uppsala, Sweden) was used for measurement of the IgG concentration in the top and bottom phases to carry out the analysis. Quantification was done by using a porous protein A affinity column from Applied Biosystems. The IgG concentration was determined from a calibration curve obtained using Gammanorm IgG as a standard.

The Bradford method was used to determine the total protein concentration using a Coomassie reagent from Pierce (Rockford, IL). The standard for protein calibration was made with bovine gamma globulin. Absorbance was measured at 595 nm in a Spectra Max 340PC microplate reader from Molecular Devices (Sunnyvale, CA).

Zeta potential measurements were performed with a Zetasizer Nano ZS system from Malvern (Malvern, UK). Particle suspensions were diluted to 0.005 wt% with different pH solutions of 10 mM KNO₃ ranging from pH 3 to 12. The zeta potential was calculated using the Smoluchowski equation. Hydrodynamic diameter measurements were performed with a Zetasizer Nano ZS system from Malvern. Particle suspensions were diluted to 0.005 wt% with Milli-Q water and then used for measurements.

Magnetic measurements were carried out using an 880 vibrating sample magnetometer (VSM) supplied by Digital

The Bradford method was used to determine the total protein concentration using an A¨kta purifier system from GE Healthcare (Uppsala, Sweden) and a porous protein A affinity column from Applied Biosystems (Foster City, CA).
Measurement Systems (Lowell, MA). Measurements were performed at 20°C with a kOe range of −10 to +10.

Magnetic particle morphology characterization and magnetic core size estimation studies were carried out using transmission electron microscopy (TEM). The samples were prepared by diluting particle suspensions using Milli-Q water followed by evaporation of diluent. After evaporation, dried particles were coated on a grid with a carbon-coated film. TEM analysis was performed in an analytical TEM Hitachi 8100 with a Rontec standard EDS detector for digital image acquisition.

Figure 1. Adsorption isotherm of EPS polymer in grams of polymer adsorbed per gram of (a) bare-MPs and (b) TEOS−MPs.

Figure 2. (a) Quantity of BSA and hIgG bound to the support under different modification stages. MP-bare (Fe₃O₄ magnetic particles), MP-TEOS (tetraethoxy silane-coated magnetic particles); MP−EPS (extracellular polysaccharide-coated particles), MP−EPS-22/8 (EPS-coated MPs modified with artificial ligand 22/8). (c) Regeneration results for MP−EPS-22/8 modified MPs in mg of protein bound per gram of MPs. (d) Quantity of IgG bound to the MP support at various time periods. Regeneration results for MP−EPS-22/8 modified.
3. Results and discussion

3.1. FucoPol production

FucoPol was produced by culturing Enterobacter A47 on glycerol under the typical cultivation conditions previously described by Alves and colleagues [23] and Torres et al. [22]. The culture attained a maximum CDW of 6.88 g l\(^{-1}\) at the end of the exponential growth phase (approx. 12 h) and a maximum EPS concentration of 5.25 g l\(^{-1}\) at the end of the cultivation run (94 h). As expected, the viscosity of the culture broth increased from an initial value of 1.15 to 1.77 \(\times\) 10\(^3\) mPa s at the end of the run. This increase in the apparent viscosity of the broth, as well as the development of shear thinning behaviour, was a consequence of the increasing polymer concentration in the broth. The compositional analysis revealed that the EPS produced was composed of fucose (31% mol), galactose (27% mol), glucose (31% mol) and glucuronic acid (12% mol), which was within the values reported for the typical FucoPol sugar composition [24]. As expected, the polymer also contained acyl group substituents (pyruvyl, succinyl and acetyl) that accounted for 25 wt.% of the polymer’s dry mass. The presence of glucuronic acid, as well as pyruvyl and succinyl substituents, confers FucoPol an anionic character [24].

3.2. Adsorption of FucoPol onto magnetic particles

The amount of EPS able to coat the surface of magnetic particles was studied. The results of the adsorption isotherm on bare-MPs are shown in figure 1a and those on TEOS–MPs are shown in figure 1b. The maximum quantity of EPS coating on bare MP- and TEOS-coated MPs was 0.29 mg g\(^{-1}\) of particles and 0.431 mg g\(^{-1}\), respectively. After successful coating of MPs using polymers, it is also important to keep in mind that the actual coating on the surface is not uniform and homogeneous, which exposes the reactive iron oxide particle to interactions with other particles while using and causing non-specific adsorption of the support. The stability and inertness results of EPS-coated MPs provide the best opportunity to use these particles for the ATPE process with further functionalization using artificial ligand or target specific organic compounds such as boronic acid.

3.3. Extracellular polysaccharide-coated magnetic particle performance in antibody purification

FucoPol provides a coating that acts as an excellent support for ligand modification of magnetic particles. This polymer satisfies dual benefits by providing a coating which is superior...
to avoid binding of undesired proteins as well as having a base which provides effective ligand functionalization. After FucoPol-coating BSA (the model protein used to study non-specific binding), the binding capacity reduced from 82 to 10 mg BSA g$^{-1}$ MPs. To have improved IgG capture capability the primary need is to have a sufficient number of functionalized groups on the surface of the polymer-modified MPs. FucoPol provides an efficient functionalization property in order to have a higher number of ligand groups on its surface, leading to maximum IgG binding capacity of 145 mg g$^{-1}$ support ($Q_{\text{max}}$) with a binding affinity constant of $4.3 \times 10^4$ M$^{-1}$.

The produced MPs were first tested for binding pure solutions of hIgG and BSA. The MP samples’ binding capacities at different stages of modification, i.e. MP–bare, MP–TEOS, MP–EPS and MP–EPS-22/8, are shown in figure 2a. Bare iron oxide (MP-bare) particles bound 40 mg BSA g$^{-1}$ MPs, 35 mg hIgG g$^{-1}$ MPs, whereas silica-coated (MP-TEOS) MPs bound slightly higher amounts, namely 82 mg BSA g$^{-1}$ MPs and 50 mg hIgG g$^{-1}$ MPs. The non-specific binding of these MPs was considerably reduced after coating with EPS to levels of 10 mg BSA g$^{-1}$ MPs and 15 mg hIgG g$^{-1}$ MPs. This reduction in non-specific binding is one of the important reasons behind the choice of the EPS polymer for coating. The MPs coated with the biospecific ligand 22/8 showed IgG binding up to 120 mg g$^{-1}$ of MPs, whereas BSA binding remained low, in the range of 8–10 mg g$^{-1}$ of MPs. The final MP–EPS-22/8 particles were then studied for static binding capacity using partition equilibrium experiments with pure solutions of hIgG.

Figure 4. Pure IgG extraction parameters in PEG/dextran systems with increasing salt concentration for (a) EPS-coated MPs, (b) EPS-22/8-coated MPs. (c) Partition coefficient for EPS-coated MPs; (d) partition coefficient for EPS-22/8-coated MPs. (Online version in colour.)

Figure 5. Electrophoresis gel under denaturation conditions to verify the binding capacity as well as the best elution conditions for IgG from the EPS-coated MPs modified with ligand 22/8 (MP–EPS-22/8) by direct magnetic separation and the ATPE method. LMW, low molecular weight; load, loading sample of the crude extract incubated with the adsorbent; Top, upper phase of the ATPE system; Bottom, bottom phase of the ATPE system; W1, first wash with binding buffer – 50 mM phosphate buffer at pH 8; E1, first elution with 50 mM glycine–NaOH buffer at pH 11; E2, second elution; D-W1, first wash with elution buffer by the direct method; D-E1, first wash with elution buffer by the direct method; D-E2, second wash with elution buffer by the direct method.
The adsorption isotherm of hIgG onto MP–EPS-22/8 was fitted using a Scatchard plot (figure 3a,b) yielding an affinity constant ($K_a$) of $4.3 \times 10^4 \text{M}^{-1}$ and a theoretical maximum capacity ($Q_{\text{max}}$) of 410 mg hIgG adsorbed g$^{-1}$ MPs. For the control MP–EPS, the determined theoretical maximum capacity was 2.6 mg hIgG adsorbed g$^{-1}$ MPs (figure 3c,d).

After preliminary studies with pure solutions of hIgG and BSA, the MP–EPS-22/8 supports were tested with a CHO cell culture supernatant by a direct magnetic separation method, in order to verify the applicability of the magnetic supports to capture antibodies from complex mixtures. The elution samples were analysed by BCA assay as well as by protein-A HPLC to quantify the amount of antibody. From the results, it was observed that, in the direct magnetic separation process using a complex crude mixture, the magnetic supports also retain their antibody binding capability and bound 122 mg hIgG g$^{-1}$ of MPs.

The partition of pure hIgG in PEG/dextran ATPS supplemented with EPS-coated MPs was also evaluated. According to the results shown in figure 4a, the addition of salt has little effect on hIgG partitioning with MPs. The salt concentration was studied for 100, 200, 300, 400 and 500 mM of NaCl. It was observed that by increasing salt concentration there is a decrease in the concentration of hIgG in the lower dextran phase and a simultaneous increase in its concentration in the upper PEG-rich phase. After the ATPE step, the particles were washed with washing buffer (50 mM phosphate at pH 8) and bound proteins were eluted using 50 mM glycine–NaOH at pH 11. During the assessment of binding of pure hIgG, the bound and eluted antibody was monitored by the BCA method. The results are shown in figure 4b for EPS–MPs (control) and EPS-22/8 MPs, respectively. Crude hIgG partitioning and hIgG binding results for the first cycle of extraction using EPS-22/8 MPs are shown in figure 4c along with partition coefficient results (figure 4d). In the case of crude hIgG purification, a sequential rise in the partition coefficient was observed from 0.11 to 0.48 with an increase of salt concentration from 100 to 500 mM. The purity of both the upper and lower phases as well as elutions from the MPs was analysed by SDS–PAGE (figure 5).

3.4. MP–EPS-22/8 characterization for size, morphology, magnetization and zeta

Particle size plays an important role in phase segregation as well as in the performance of MPs in an ATPS. Amination methods also have an impact on MP properties, leading to particle aggregation [1], density and organization of the resultant surface functional groups which can be easily overcome by using the triphasic reverse emulsion technique described in [17].
The modifications using EPS polymer increase the hydrodynamic diameter of the particle. As shown in figure 6f, the average hydrodynamic diameter for EPS-coated MPs is around 1100 nm. The average hydrodynamic diameter for bare MPs varies in the range of 260–265 nm. In the case of the particles coated with TEOS, there is a slight rise in the average hydrodynamic diameter to 365–375 nm. The MPs with 22/8 have an average diameter in the range of 970–995 nm, even though some particles of size up to 3000 nm are also observed in the suspension.

The morphology of the particles determined with TEM shows the formation of larger agglomerates when the particles were coated with polymers. It can be seen that the diameter of each individual magnetic core is in the range of 10–20 nm (figure 6c,e). This might be explained by the non-covalent interactions between the coating biopolymers of neighbouring nanoparticles. According to figure 6c,e, it is possible to conclude that the supports synthesized are not uniform in size and present a high polydispersity.

As shown by the data in figure 6b, it was observed that the zeta potential values of particles varies between −2 and −20 with variation of pH values between 3 and 12. EPS-coated MPs have a zeta potential in the range of −2.5 to −17, which shows a higher variation above pH 10. For EPS-22/8-MPs at a lower pH of 3, the zeta potential value was −7.3 mV, which increases gradually until pH 12, when the zeta potential value observed was −20.1. When particles are coated with the different polymers the global charge is altered according to the type of biopolymer used. Magnetic property characterization results determined using VSM are shown in figure 6g. The curves represent the reversibility and symmetry that is easily observed in the figure. The curves show the typical characteristic super-paramagnetic behaviour of the synthesized particles. In terms of saturation magnetization, the values obtained were 56.5 emu g⁻¹ for EPS–MP and 58 emu g⁻¹ for EPS-22/8-MP.

4. Conclusion

Magnetic supports with a basic core of iron oxide show superior suitability for the utilization of Fucopol, a fucose-containing EPS produced by Enterobacter A47, as a coating material for MPs. EPS was found to be a promising polymer for MP coating and modification owing to the simplified synthesis, low cost, high stability and reduced non-specific adsorption. The excellent capability of EPS-coated MPs for the covalent attachment of a synthetic affinity ligand makes these particles challenging for recovery of biomolecules, specifically for antibodies. EPS-coated MPs also show applicability for use in the integrated process technology that joins the magnetic separation process with ATPE for the purification of human antibodies. The ATPS composed of 8% PEG and 5% dextran afforded a 90% yield in the presence of EPS-22/8-coated MPs. The magnetic supports can be effectively used five times with partial reduction in binding capacity.


References

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