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Isolation and purification of blood group antigens using immuno-affinity chromatography on short monolithic columns

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ABSTRACT

Monolithic columns have gained increasing attention as stationary phases for the separation of biomolecules and biopharmaceuticals. In the present work the performance of monolithic convective interaction media (CIM®) chromatography for the purification of blood group antigens was established. The proteins employed in this study are derived from blood group antigens Knops, JMH and Scianna, equipped both with a His-tag and with a V5-tag by which they can be purified. In a first step a monoclonal antibody directed against the V5-tag was immobilized on a CIM® Disk with epoxy chemistry. After this, the immobilized CIM® Disk was used in immuno-affinity chromatography to purify the three blood group antigens from cell culture supernatant. Up-scaling of the applied technology was carried out using CIM® Tubes. In comparison to conventional affinity chromatography, blood group antigens were also purified via His-tag using a HiTrap® metal-affinity column. The two purifications have been compared regarding purity, yield and purification speed. Using the monolithic support, it was possible to isolate the blood group antigens with a higher flow rate than using the conventional bed-packed column.

1. Introduction

The aim of this study was to establish a tailor-made immune affinity chromatography for the rapid isolation and purification of blood group antigens from cell culture supernatants. The term "blood group antigen" (BGA) refers to a set of proteins that are found on the surface of human red blood cells or platelets. As already implied by their name, a commonness of the members of this group is the expression of antigenic activity. Apart from this, red blood cell and platelet antigens can be extremely diverse. The functions assigned to these proteins may vary from membrane structure, adhesion, enzyme activity, and many more. Due to their immunogenic potential, the fast and reliable detection of antibodies against these proteins in a patient's serum is a challenge in immunohematology. At present time, a state of the art assay for the detection of these antibodies could make use of highly purified recombinant proteins for the capturing of such antibodies [1–8].

Due to the diverse nature of BGA and the very limited availability of BGA specific antibodies, a purification platform independent of the nature of the individual antigens is highly desired. Different modes of liquid chromatography, like ion exchange, size exclusion, and especially affinity chromatography is used for the purification of BGA [2,6–12]. In our work, we dealt with the purification of three recombinant proteins derived from blood group antigens Knops, JMH and Scianna. In a conventional approach, blood group antigens as well as other biomolecules are purified using chromatographic techniques based on bed-packed columns. These techniques are limited by high pressure drop, intraparticle diffusion, low flow rates, as well as flow rate dependent dynamic capacities, resulting in increased process time and recovery liquid volume and complicated scale-up [13–15]. To overcome these limitations, the employment of new stationary phases is a promising approach. Therefore, we developed a chromatographic method based on CIM (convective interaction media) monolith for the rapid purification of three recombinant proteins derived from blood group antigens Knops, JMH and Scianna. Over the last few years, monolithic convective interaction media chromatography has become a useful tool in the separation of biomolecules and biopharmaceuticals [16–25]. Porous monolithic disks permit fast binding kinetics and a high separation speed that allows a fast development of chromatographic methods. This is why affinity chromatography on short monolithic columns seems to be an attractive method for overcoming many critical disadvantages of conventional affinity chromatography. The monolithic supports are produced from a copolymer of glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA). They have the advantages of high capacity, high flow rate, and lower pressure drop as well as chemical flexibility for ligand attachment. Due to the highly porous character of monolithic supports, where the pores are interconnected, a network of channels is formed. Thus,

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the molecules are solely transported by convection. This results in a better mass transfer mechanism between the mobile and stationary phase, and the transport of the sample is not limited by diffusion. In addition the resolution and dynamic binding capacity of the medium do not change with the flow rate [13,19–22,25–30].

Over the last decade, this special class of chromatographic supports has been intensively studied. Monoliths of different modes have been used successfully in the separation of biomolecules in analytical as well as in preparative scale. They provide the support for the immobilization and isolation of peptides [16,31–34], proteins [14,32,33,35,36], enzymes [33,37–41], viruses [42,43] and plasmid DNA [44–46] as well as antibodies or antigens for immuno-affinity chromatography [32,35,47–50]. Especially their use in affinity chromatography to separate antibodies, enzymes, tagged proteins, etc. has been reviewed by several authors [32,34,35,51–55].

For the purification of BGA, the most efficient isolation can be realized using immuno-affinity modes. This very popular separation technique is based on the biocomplementary interactions between antibodies and either their specific antigen [56,57] or ligands of general specificity like Proteins A, G, and L [23,50,58]. The application of immuno-affinity chromatography with immobilized monoclonal antibodies on CIM® Disks was described by Schuster et al. [47].

In this present work, CIM® Tubes with epoxy chemistry were used as a support for the immobilization of the monoclonal anti-V5 antibody. The prepared immuno-affinity tubes were used for the preparative isolation of the three blood group antigens Knops, JMH and Scianna directly from cell culture supernatant via their V5-tag. To compare this tailor-made immuno-affinity chromatography with a conventional affinity chromatography, the blood group antigens were also purified via their His-tag using a HiTrap® metal-affinity column. The two purifications have been compared regarding purity, yield and purification velocity. Using the monolithic support, it was possible to isolate the blood group antigens with higher purity and higher flow rates resulting in faster processing time than using the conventional bed-packed column.

2. Materials and methods

2.1. Chemicals

The monoclonal antibody and the proteins derived from blood group antigens Knops, JMH and Scianna were raised in our laboratory. Chemicals for buffer preparation and ELISA were purchased from Sigma–Aldrich (Steinheim, Germany). All these chemicals were of per analysis quality. All buffers and protein solutions were prepared in demineralized H_2O (ddH_2O) (Arium, Sartorius, Göttingen, Germany).

2.2. Creation of the anti-V5 hybridoma cell line

Creation of the hybridoma cell producing the monoclonal antibody against the V5 tag was performed by Eurogentec Corporation (Seraing, Belgium). Immunization was carried out with a synthetic peptide fused to KLH.

2.3. Construction of BGA proteins

All the wild type forms of the proteins described in this study are membrane bound, either via a GPI anchor (JMH, Genbank Nucleic Acids NM_003612) or via a transmembrane domain (Scianna, NM_018538; Knops, NM_000651). The soluble forms of Scianna and JMH were produced by fusion of the nucleotide sequence coding for the extracellular part of the protein to the V5 tag and HIS tag, where the V5 tag is separated from the BGA fragment by a 22 aa spacer. Production of the soluble Knops protein was accomplished by fusing the 4th long homologous repeat (LHR) domain (Base 4168-5904) to the spacer–V5/HIS sequence already known from the soluble JMH and Scianna constructs. After lentiviral transduction, the protein was produced in HEK cells.

2.4. Antibody immobilization

Standard CIM® Epoxy Disks from BIA Separations (Ljubljana, Slovenia) were used. The disk dimensions were 12 mm × 3 mm corresponding to a bed volume of 0.34 mL and a mean pore size of 675 nm.

Prior to antibody immobilization the disks were prepared by flushing with 10 mL ethanol, 10 mL ethanol/ddH_2O, 10 mL ddH_2O, and finally with 15 mL immobilization buffer (0.1 M sodium carbonate, 0.5 M NaCl, pH 9.3) at a flowrate of 1 mL/min.

The equilibrated disks were then flushed with 2 mL antibody solution, dissolved in immobilization buffer (5 mg/mL), and afterwards incubated for 24 h at room temperature. After incubation, the excess antibody was washed out and collected for the determination of coupling efficiency.

The remaining epoxy groups on the CIM® medium were inactivated by flushing the disks with 5 mL 1 M ethanolamine.

2.5. Chromatography procedures

After harvesting, cell culture supernatants were centrifuged at 1000 × *g* for 15 min, filtrated over a 0.7 μm and a 0.2 μm filter and then bisect. For the immuno-affinity chromatography via monoliths, the supernatants were adjusted to pH 7.8. After equilibration of the chromatographic tube with PBST, samples were applied at a flow rate of 16 mL/min. After washing with PBST until the baseline was reached, elution was carried out using a buffer composed of 50 mM NaPP, 250 mM NaCl, 0.05% Tween-20, pH 11.5. Elution fractions were neutralized with 1 M Tris/HCl, pH 6.8. The filtrated supernatants for the affinity chromatography using HiTrap® were desalted against 50 mM Tris/HCl, 150 mM NaCl, 20 mM imidazole, pH 8. After equilibration of the metal-affinity column with 50 mM Tris/HCl, 150 mM NaCl, 20 mM imidazole, pH 8.0, samples were applied at a flow rate of 2.5 mL/min. After intense washing, elution was carried out using a buffer composed of 50 mM Tris/HCl, 150 mM NaCl, 500 mM imidazole, pH 8. Afterwards, the samples from both chromatographic methods were desalted against PBS, pH 7.4.

2.6. Antibody and protein analytics

An *enzyme-linked immunosorbent assay (ELISA)* was employed to examine the yield of the individual purification steps. 96-well microtiter plates were coated overnight with anti V5-antibody (Serotec MCA1360, 1:200) and then blocked with 2% BSA in phosphate buffered saline (PBST) for 30 min at RT. After washing, samples were added to the plates (diluted in BSA/PBST) and incubated for 4 h at RT. After a further washing step, a polyclonal rabbit-anti HIS:HRP antibody (Antibodies-online, ABIN195463; dilution 1:7500) was added to each well. After incubation for 1 h, the wells were washed again and the peroxidase substrate solution (TMB) was added. After 15 min, the reaction was stopped by the addition of stop solution (HCl, H_2SO_4, H_2O) and the absorbance of each well was measured at 450 nm in a BioTek Powerwave® microplate Reader.

Where possible protein concentrations were also quantified by Bradford assay, in good accordance with the concentrations determined by ELISA. Absorbance was measured at 592 nm using a BioTek Powerwave® microplate Reader. BGG was used as standard.

2.7. On-Chip CE

The On-Chip Capillary Electrophoresis (CE) allows for fast analysis of sample purity with the sensitivity of a Coomassie staining. Automated peak integration of obtained electropherograms yields an easily standardizable procedure for the comparison of different chromatography techniques. For comparison of different runs, visualization of the data in form of a simulated SDS-PAGE lane is generally preferred.

ON-Chip CE was performed on an Agilent 2100 Bioanalyzer according to the very detailed manuscript of the manufacturer.

3. Results and discussion

In order to develop a tailor-made fast immuno-affinity chromatography for the isolation and purification of BGA from cell culture supernatant, monolithic CIM® Disks with epoxy chemistry were investigated for the immobilization of a monoclonal antibody directed against the V5-tag. As mentioned above, three proteins were chosen as targets that were to be purified. To compare monolithic convective interaction media-chromatography with the conventional affinity-chromatography the blood group antigens were purified via their V5-tag just as via their His-tag. The conventional chromatography was already established but we had to arrange the purification using immobilized monolith columns.

3.1. Optimization of the antibody immobilization

The antibody immobilization is an important matter in our purification process. An antibody, produced in our laboratory, was chosen as a test antibody to optimize the immobilization procedure for CIM® Disks with highly active epoxy groups. These groups react with any amino group residue of the affinity ligand, in our case the amino group residue of the antibody, to form a covalent linkage. The epoxy chemistry as well as the highly porous character of the monoliths should allow accomplishing the immobilization in a single step under mild conditions even in the absence of spacers [50]. A prerequisite for an efficient coupling procedure via epoxy groups is the delivery of the ligand that is to be coupled in an appropriate buffer. In our case, a fairly basic buffer with elevated ionic strength was able to meet these demands. Thus, a 0.2 M sodium carbonate buffer containing 0.5 M NaCl, pH 9.3 was used as immobilization buffer. While standard protocols for the immobilization of ligands via epoxy groups is performed under mild basic conditions (pH 8) [59] coupling at higher pH values turned out to be advantageous, especially concerning the coupling time. With the conditions described, we were able to reduce the incubation time from 6 to 8 days to as low as 1–2 days. Eventually, this resulted in a coupling procedure combining the comfort of the epoxy coupling and the speed of other, more laborious techniques [60]. To determine the optimal coupling technique, the antibody immobilization was carried out using different immobilization procedures under static and dynamic conditions. In one approach, the antibody solution was pumped through the CIM® Disk. Subsequently, the disk was stored for 7 h at 30 °C and afterwards a fresh antibody solution was pumped through the CIM® Disk, and the disk was stored for further 14 h at 30 °C. An alternative was to store the CIM® Disk after loading with antibody solution for 2 days at room temperature. In an alternative approach, antibody immobilization was carried out under dynamic conditions in a flow-through mode closed circuit for 16 h. The amount of antibody coupled to the CIM® Disks was determined by the material balance (amount of antibody in the supernatant before and after immobilization) measured by enzyme-linked immunosorbent assay. In contrast to the enzyme immobilization in flow-through mode of Delattre et al. [40], it has been obtained that the antibody immobilization yield was much better using static conditions. Consequently, the immobilization procedure was carried out as a one step process at static conditions by room temperature. This procedure, as described in Section 2.2, was selected for further work. The binding capacity of the epoxy CIM® Disk was then estimated around 1.2 mg of monoclonal antibody per CIM® Disk. The optimized method of antibody immobilization successfully developed on CIM® Disk was applied to larger CIM® Tube with the same efficiency. CIM® 8 mL Tubes enable the BGA purification in a preparative scale.

3.2. Development of immuno-affinity chromatography

As a first step in the development of a tailor-made fast immuno-affinity chromatography the immobilized CIM® Disks were used in the investigation of the effect of pH on the performance of the protein isolation from cell culture supernatant. Comparison of buffers of different compositions and pH values were performed for the optimization of the elution conditions using a 50 mM citrate buffer (pH 4.0) and a 50 mM phosphate buffer (pH 10.5, 11.0, 11.5). The results suggest that the pH values did affect the elution achievement. Performing an acidic elution, no protein could be eluted. In contrast, an elution at elevated pH values led to success. While no significant elution of the protein could be observed at pH 10.5, 11.0 and 11.5 protein elution was achieved. Whereas at pH 11.0 the elution peak showed a massive tailing, the release of the target protein at pH 11.5 resulted in a peak with sufficient sharpness. The highest protein yield was achieved using 50 mM sodium phosphate, 250 mM NaCl, 0.05% Tween-20, pH 11.5 as an eluting buffer. Consequently, this buffer system was selected for further work. It was possible to perform an up-scale of protein isolation using CIM® 8 mL Tubes. In order not to impair the integrity both of the immobilized antibody and of the target protein, elution at pH values higher than 11.5 has not been tested.

Furthermore, the influence of flow-rate on protein adsorption was also studied. Experiments were performed employing flow rates in the range of 0.17–4.0 mL/min and in later experiments flow rates up to 16 mL/min were tested (data not shown). The results showed no dependency on the flow-rate. Therefore we can conclude that high flow rates do not impede the isolation of BGA using monolithic CIM®-chromatography.

3.3. Comparison of the chromatography techniques

As a starting point of this study, the immuno affinity procedure had to be optimized, requiring a reliable method for coupling antibodies to the monolithic chromatography using the small CIM® Disks. Based on a previous study on how to efficiently immobilize a commercially available antibody on these media (results not shown), immobilization of the anti V5 antibody was carried out under the conditions that have shown highest efficiency (see Section 2.2). On the basis of such prepared CIM® Disks the immuno-affinity chromatography performance was examined employing the protein derived from Knops blood group antigen. The developed method was transferred to CIM® 8 mL Tube giving the same results. After this, it was easy to confer the purification in preparative scale on the proteins originated from Knops, Scianna and JMH blood group antigens.

Based on the previous results the three derived proteins were separated on CIM® 8 mL Tube using the appropriate method and conditions described in Section 2.3. The product purity of the isolated proteins was analyzed by ON-Chip CE with a visualization of the data in form of an SDS-PAGE lane with a sensitivity of a Coomassie Brilliant Blue staining. The protein amounts were measured by Bradford assay.

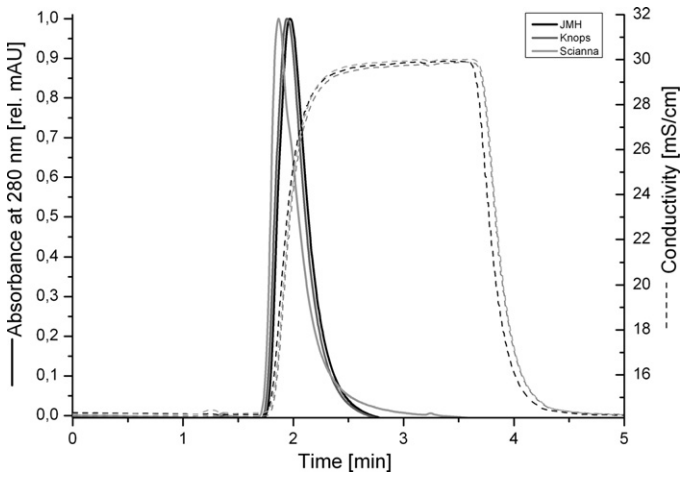

Fig. 1. Separation of BGA proteins from cell culture supernatant on CIM Tube® at a flow rate of 16 mL/min. BGA proteins were eluted by 250 mM NaCl in 50 mM NaPP, pH 11.5.

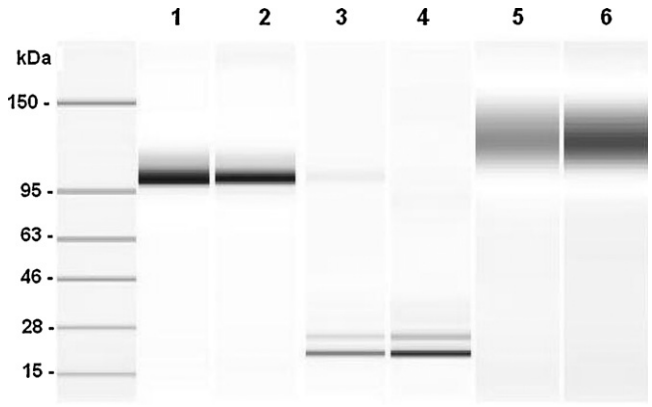

Fig. 3. Visualization of the On-Chip CE data in form of SDS-PAGE lanes. Lanes show the eluted protein fractions after affinity chromatography using HiTrap® column and after immuno-affinity chromatography using CIM® Tube (Lanes: 1, Knops protein, 128 kDa (CIM® Tube); 2, Knops protein, 128 kDa (HiTrap®); 3, JMH protein, 103 kDa (CIM® Tube); 4, JMH protein, 103 kDa (HiTrap®); 5, Scianna protein, 19 kDa (CIM® Tube); 6, Scianna protein, 19 kDa (HiTrap®)).

For the assessment of media performance, harvests of cell culture supernatants of all three proteins were divided into halves, with one half being purified via monolithic media and the other half, as a representative for packed bed chromatography, via HiTrap columns.

Isolation of the three proteins Knops, JMH and Scianna by CIM® method is shown in Fig. 1.

The chromatogram verified that the binding and elution of all three proteins was specific. In all three cases contaminating proteins were successfully removed and therefore no other peaks could be detected in the elution fractions. The elution of the bound proteins was completed after 2.5 min (Fig. 1). To compare, this purification process was also carried out in preparative scale using the standard protocol described in Section 2.4. The purification of the BGA based on HiTrap® column takes 7.5 min (Fig. 2). The target proteins bind to the column and can be eluted with 150 mM NaCl. Using the tailor-made immune-affinity chromatography BGA proteins can be purified three times faster because of the higher applicable flowrate of 16 mL/min. This method provides also very high product purity.

By On-Chip CE (see Fig. 3) no other bands but the target protein bands could be detected. Just one single band of the proteins derived from Knops (lane 1) and JMH (lane 5) were obtained. The two protein bands in the range of 20 kDa belong to the Scianna protein. Also this isolated protein fraction (lane 3) offered the pure protein. The 95 kDa protein band seems to be an aggregate.

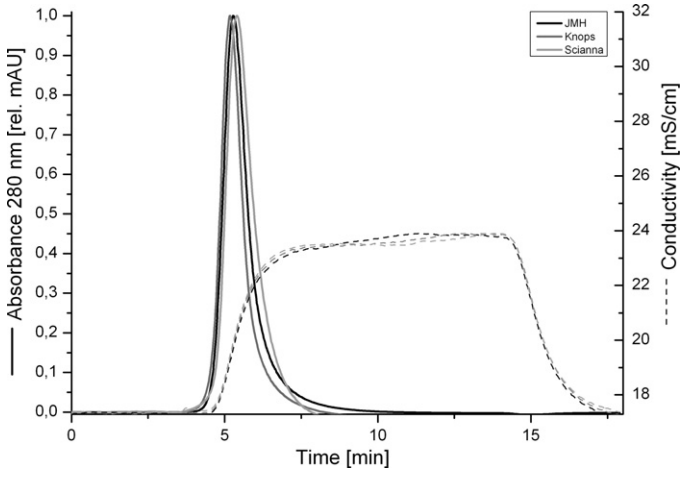

Fig. 2. Separation of BGA proteins from cell culture supernatant on a HiTrap® column at a flow rate of 2.5 mL/min. BGA proteins were eluted by 150 mM NaCl in 50 mM Tris/HCl, pH 8.0 containing 500 mM imidazole.

In general, employing the conventional chromatography technique not the same purities as possible in immuno-affinity chromatography were achieved (see Table 1). Nevertheless, we can allude to an excellent purity in both cases. As can be seen from Fig. 3, the proteins from Knops (lane 2), JMH (lane 6) and Scianna, showing almost two to three bands (lane 4), were already isolated in pure form.

Both chromatography techniques provide the isolation of pure protein. But much better performance in regard to selectivity and capacity has been observed with the monolithic CIM® Tube. Protein concentrations in the chromatographic fractions were measured by Bradford assay as described in Section 2.6. Table 1 shows the eluted protein amount achieved by both chromatography techniques. The CIM® Tube provided higher amount of the eluted protein in all cases. With all three proteins, the eluted amount was up to 50% higher when using monolithic supports instead of gel beads.

These results indicate that high productivity of purification process can be achieved using CIM® Tube, especially in the isolation of the proteins from Knops and Scianna.

After purification via CIM® Tubes, the recovery after performing a run was far above 90% based on the estimation of protein concentration in the crude cell supernatant. The observed recoveries of red blood cell and platelet antigens purified via their His-tag by means of a standard protocol is surprisingly low and can presumably be improved in further experiments. However, even with an optimized protocol the recovery seen with the monolithic media cannot be exceeded.

It should be noted that flow rates using CIM® Tube were 16 mL/min and thus six times faster than flow rates employing the HiTrap® column (2.5 mL/min). Due to this fact, the isolation can be carried out rapidly, which results in higher productivity.

Table 1
Purification of Knops, JMH and Scianna protein using CIM® Tube and HiTrap® column: eluted protein amount in the chromatographic fractions. Parenthesized are the protein purities determined by Bioanalyzer.

	Protein amount [mg] (purity [%])	
	CIM® Tube	HiTrap®
Knops	13.6 (95.3)	8.7 (97.2)
JMH	65.5 (97.6)	60.1 (92.2)
Scianna	27.8 (97.8)	20.0 (95.8)

4. Conclusion

A tailor-made monolithic based immuno-affinity chromatography was developed for the isolation and purification of blood group antigens. By covalently coupling an anti-V5 antibody onto the monolith surface, the three proteins derived from the blood group antigens Knops, JMH and Scianna could be captured from crude cell culture supernatant. The antibody immobilization and the purification performance were optimized and the application of this method was studied. In all cases the purity of the isolated proteins was very high. These results were comparable to that of the proteins purified by conventional chromatography method based on HiTrap® column. But our new procedure using CIM® Tubes provides a way of isolating the proteins is much faster and more efficient than the conventional chromatography.

Clearly, the CIM® Disks and Tubes have advantages over conventional materials in terms of speed and throughput. They may considered not to be suited for applications, where financial restrictions are a major issue. In this case, the cheaper packed bed system could be the better choice, although their lower throughput and lower binding capacity may quickly result in an economically inefficient usage.

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