Preparation and characterization of large format macroporous cryogel discs for use in affinity chromatography and biotechnological applications.

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Abstract

We have prepared and evaluated larger format phage-bound epoxy-cryogel columns in order to increase the yield of bound target. Freezing thermograms showed that larger column formats (2,5 cm-5 cm diameter) are not usable due to irregular polymerization phenomena. Preparing thin discs of 0,5 cm height with similar diameter proved to be an excellent alternative. Discs could be stacked and run in a chromatographic set up. In this way we could increase the matrix volume, ligand binding capacity and finally the yield of bound target. By increasing the column volume about 7 fold, we observed a twelve fold increase of ligand density and a 7 fold increase in the yield of protein recovery in a column where phages were attached without spacer and a 10 to 34 fold increase in a spacer column, depending on the spacer used.

Keywords

Cryogel disc, peptide-displaying phage ligand, affinity chromatography

Introduction

Supermacroporous cryogels are a novel generation of chromatographic stationary phases that can be used for purification of particulate containing feed stocks without prior steps in the down-stream processing [1,2]. The supermacroporous structures with interconnected pores of up to 100 μm allow purification of materials from different size without risk of clogging the matrix. Very small molecules, e.g. metals, toxins, hormones, up to larger structures such as proteins, plasmids, viruses and even intact cells can be processed on the cryogel columns. Due to the big pore structures inside the gel also high flow rates can be applied such that large batches of sample can be processed in a reasonable time frame. Next to these structural advantages, cryogels are low cost and can be readily prepared in different formats such as monolithic columns, thin sheets, beads or discs. Big pore structures inside the cryogel however also means that only a small amount of polymer, 3 – 4% of the total volume [3] is available for anchoring ligands. This has a direct influence on the binding capacity of such columns. Preparing larger sized monolithic columns is not straightforward due to restrictions correlated to the cryo-polymerization procedure. Cryo-polymerization is highly dependent on the freezing temperature. The temperature should be as constant as possible over the whole gel to insure equal freezing over the whole gel. By preparing larger column formats, a difference in the freezing temperature is observed as shown by the freezing thermograms, resulting in uneven formation of pores. To deal with this problem we focused on the preparation of monolith sheets/discs instead of columns. The sheets are casted in a glass mold and after polymerization discs are cut out. Discs are prepared with diameters varying from 0.5 cm diameter ($\varnothing$) up to 1.2 cm $\varnothing$. Discs made of poly(2-hydroxyethyl methacrylate) (pHEMA) have been used with different protein ligands in the range of 0.5 cm $\varnothing$ to 1.2 cm $\varnothing$: e.g. IgG was purified from human plasma on a 0.5 cm $\varnothing$ thiophilic pHEMA cryogel disk [4] or proteins could be purified by 0.8 cm $\varnothing$ – 2.5 cm $\varnothing$ pHEMA cryogel discs with reactive dye ligands [5,6] but also plasmid DNA was purified on 0.8 cm $\varnothing$ – 1 cm $\varnothing$ pHEMA cryogel discs with different coupled ligands [7,8]. Apart from biological materials described above, also metals could be recovered on cryogel discs. Cadmium was recovered on a 0.8 cm $\varnothing$ reactive dye-pHEMA cryogel disc [9] while uraniumoxide was trapped on a 1 cm $\varnothing$ glutamic acid-pHEMA cryogel disc [10]. Acrylamide cryogel discs ($\varnothing = \pm 2.4$ cm) have been successfully used for the continuous production of monoclonal antibodies from a hybridoma cell culture which grows in the discs [11].

In the present work we have focused on the preparation and characterization of polyacrylamide-epoxy based cryogel discs with an ≥ 2.5 cm $\varnothing$. Discs with different acrylamide concentrations were made and compared in function of ligand binding and target binding capacity. As in earlier work [12] we determined the beneficial effect on the yield using spacers to immobilize selected phage, we here will combine upscaling the column format by using larger discs columns in combination with the introduction of spacers to obtain a higher ligand binding allowing to further optimize the yield of the target of interest.

Materials and Methods

Materials

Allyl glycidyl ether, iminodiacetic acid and 1,6-diamino hexane were purchased from Sigma-Aldrich (Bornem, Belgium); acrylamide, methylene-bis-acrylamide, TEMED, ammonium persulfate, glutaraldehyde, ethanolamine and iminodiacetic acid (IDA) were from Sigma (St.Louis, Mo); C10/10, XK16/20, XK26/20, XK50/20 columns and Äkta Pure chromatographic system are from GE Healthcare (Uppsala, Sweden);

Vernier stainless steel temperature probe and Logger Pro software are from Vernier (Beaverton, OR).

Experimental methods

Preparation of Epoxy-cryogel by cryogenic polymerization

Epoxy-cryogel columns with a total monomer concentration of 6% or 7,5% are prepared according to published protocols [13]. Briefly, acrylamide (Aam) and methylene-bis-acrylamide (Bis-Aam) are mixed in a molar ratio of Aam/Bis-Aam= 5. Allyl glycidylether (AGE), providing reactive epoxy groups, is added to Aam/AGE in a molar ratio of 10 to obtain a cryogel with available epoxy groups for further coupling strategies. A 10% ammonium persulfate (APS) solution in water and TEMED in a final concentration of respectively 1% (V/V) and 0,156% (V/V) are added to the mixture. The polymer mixture is poured in different column formats (GE Healthcare, Uppsala, Sweden) and polymerized at -12°C for 20 hours. After polymerization the columns are thawed at RT and extensively washed with MilliQ-water (MQ-water) to remove all nonpolymerized material. Cryogel discs are prepared in similar conditions as above by pouring the polymer mixture in a glass mold (5 mm spacer). After polymerization and thawing the cryogel was cut in circular discs, 2.6 cm or 5 cm Ø. The discs are extensively washed with MQ-water and stacked in a column with corresponding diameter.

Preparation of iminodiacetate-cryogel

To determine the ligand density, iminodiacetic-cryogels (IDA-cryogel) are prepared [13]. Briefly, the epoxy-cryogel columns (1 cm – 1.6 cm Ø) are washed with 60 ml 0.5M Na₂CO₃ followed by a wash with 60 ml 1M Na₂CO₃ both at 1 ml/min. Next, 50 ml 0.5M IDA in 1M Na₂CO₃ pH=9.6 is recirculated over the column at 0.5 ml/min for 20 hours at RT. The column is washed with 60 ml 1M Na₂CO₃ at 1 ml/min followed by extensive washing with MQ-water until neutral pH. In a similar way the stacked disk columns (2.6 cm – 5 cm Ø) are washed with respectively 90 ml 0.5M Na₂CO₃ and 1M Na₂CO₃ at 1 ml/min, followed by recirculation of 100 ml 0.5M IDA in 1M Na₂CO₃ pH=9.6 at 0.5 ml/min for 20 hours at RT. Then the disk column is washed with 90 ml 1M Na₂CO₃ at 1 ml/min followed by extensive washing with MQ-water until neutral pH. The columns are stored at 4°C until further use.

Thermograms

The freezing profiles are recorded by placing 1 or 2 Vernier temperature probes at half height in the polymerization solution. Freezing is done at -12°C . Temperature changes are measured in function of time until the temperature reaches T_f (temperature set in the cryostat). A single probe was used for 1 cm $\varnothing$ column and for the glass mold setup. Two probes were placed in the 2.6 cm and 5 cm $\varnothing$ columns: the first one near the edge of the column and the second probe in the middle of the column. Data are recorded by LoggerPro software (Vernier).

Scanning Electron Microscopy

For Scanning Electron Microscopy, the polymerized discs were cut in wedges, exposing the surface from the center of the disc to the periphery (fig.2, view A), as well as the interior (fig.2, view B). The pieces were mounted on a 2.5 cm diameter SEM support stubs with carbon stickers and carefully applied silver paint. Subsequently, the samples were sputter coated with 10 nm chromium in a Leica ACE600 coating machine. Samples were observed and images taken with a Zeiss Sigma scanning electron microscope, operated at 5 kV at high vacuum with a SE detector, or at reduced vacuum (0.16 Pa) with a VPSE detector, and a working distance of about 7 mm.

Determination of epoxy density on the cryogel

The amount of available epoxy groups for ligand coupling, is determined according to Arvidsson et al. [13]. The epoxy-cryogel is washed with MQ-water and next saturated with a 0.5M CuSO_4 solution in water at 1 ml/min at RT. Excess of Cu^{2+} is washed away with MQ-water. Bound Cu^{2+} is then eluted with 0.1M EDTA pH=7.6 and determined spectrophotometrically at 730 nm. The Cu^{2+} -EDTA complex has a $\epsilon_{730} = 46.8 \text{ M}^{-1} \text{ cm}^{-1}$.

Phage coupling on epoxy-cryogel

Filamentous M13-bacteriophages, clone HuN and HuL, are selected from phage display libraries for binding to human lactoferrin (HULF) as described earlier [14]. Phage coupling to the epoxy-column was done as described earlier [10]. Briefly, the epoxy-cryogel is washed with MQ-water at 1 ml/min followed by an equilibration step with 0.2M Na_2CO_3 at 1 ml/min. Next the phage solution in 0.2M Na_2CO_3 is recirculated over the column at 0.5 ml/min for 16 hours. After phage coupling the column is washed with 0.2M Na_2CO_3 at 1ml/min to remove unbound material. Next, the column is incubated with 0.1M ethanolamine in 0.2M Na_2CO_3 at 0.5 ml/min for 3 hours to block all remaining free epoxy groups. Finally the column is extensively washed with phosphate buffered saline (PBS) until the pH= 7.5. All steps are performed at RT. At this stage the column is ready to perform binding experiments and can be stored at 4°C until further use.

Protein binding experiments

The cryogels are housed in commercial available column hardware as mentioned in the materials section. We used columns from GE Healthcare such as C10/10, XK16/20, XK26/20 or XK50/20. These columns were optimal as they were easy to handle as they had the correct dimensions to prepare columns of different dimensions for chromatographic experiments. The epoxy-phage-cryogels are connected to an Äkta

Pure system (GE Healthcare, Uppsala, Sweden) and equilibrated with PBS buffer. Defatted human milk is diluted twice with PBS and is loaded on the column and washed with PBS to remove unbound protein. The column is then eluted with phosphate buffer–1M NaCl pH=7.5 (PB-1M). The eluted protein fractions are pooled for quantification. See table 1 for running parameters. Protein concentration of the pooled PB-1M fraction is determined based on the Folin-Ciocalteus method [15]. The yield is finally expressed as μg bound protein.

Results and Discussion

Epoxy-cryogel

Column structure

Poly-acrylamide based cryogels are prepared by cryo-polymerization at -12°C . To introduce reactive groups, allyl-glycidylether (AGE) is added as additional monomer in the polymerization solution. As evaluated earlier [12] optimal structural conditions and optimal AGE concentrations are observed at a molar acrylamide/AGE ratio of 10. These conditions are used for preparation of all further cryogel gels. Until now only 1 cm $\varnothing$ cryogels have been prepared. In order to improve the low binding capacity inherent to these gels, we now prepared larger formats and evaluated them. In first instance, columns of 1.6 cm $\varnothing$, 2.6 cm $\varnothing$ and 5 cm $\varnothing$ were prepared and compared to the 1 cm $\varnothing$ column. Freezing thermograms are recorded as we anticipated the occurrence of a temperature gradient during polymerization in the larger sized columns. If the freezing temperature is not constant over the overall column, differences in polymerization rate might influence the column structure and especially the pore size. In the 1 cm $\varnothing$ column we placed 1 temperature probe in the middle of the column, while for the 2.6 cm $\varnothing$ and 5 cm $\varnothing$ column 2 probes are used, one near the edge of the column and 1 in the middle of the column. We could clearly see that, when the diameter of the column was increased, indeed a temperature gradient over the column diameter was observed (Fig. 1a.) in contrast what happens in the 1 cm column. After the crystallization is completed in the 1 cm $\varnothing$ column, the temperature drops quickly to the fixed T_f of -12°C in accordance to earlier results [16,17]. For the 5 cm $\varnothing$ column we see a clear difference between the edge and the middle of the column. At the edge of the column the temperature drops more quickly than in the middle of the column. This can affect the polymerization rate of the cryogel resulting in an irregular pore size pattern. As can be seen in figure 1c there indeed is a clear difference in pore size at the edges compared to the middle of the cryogel, with a regular polymerization at the edges, while in the middle an irregular polymerization is observed with big gaps in the cryogel. Similar results were observed by Savina et al. [18]. In order to overcome this, larger formats were prepared by polymerizing the cryogel in a 0.5 cm glass mold. This resulted in an even better thermogram as compared to the 1 cm $\varnothing$ column (Fig. 1b). The thinner the cryogel, the more even polymerization is observed over the whole gel. The pore structure of the cryogel discs was evaluated by scanning electron microscopy (Fig. 2), which showed equal pore size over the whole cryogel disk from periphery to the center of the disk. Also in depth the pore size is equally divided. No significant deterioration of the porous structure caused by drying the cryogel discs was observed by SEM or ESEM [19]. As we prepare cryogels by the same protocol as

referred in previous paper we believe we can trust that the morphology we observed by SEM (fig. 2) is representative for the used material.

We next prepared and evaluated cryogel columns with 14 discs of 2.6 cm Ø stacked or 7 discs of 5 cm Ø stacked. During stacking a slight pressure is applied to obtain a good contact between the individual discs. The cryogels are highly elastic so the applied pressure has no influence on the separation profile as observed in earlier experiments (data not shown). Applying too high pressure on a classical bead column affects the packing showing negative effects in the separation profile or in the worst case collapse of the column.

Epoxy density on the cryogel

For all cryogel formats used, the molar ratio Aam/AGE=10. As such, an increase in epoxy groups therefore should be correlated to the volume of the cryogel and indeed the amount of available epoxy groups increased linearly with the column volume (Fig. 3) as expected. The column volumes used for a regular 1 cm and a 1.6 cm Ø column were respectively 5.5 and 11.5 ml. A 2.6 cm Ø disk column with 14 discs stacked has a column volume of ± 37 ml, the 5 cm Ø disk column with 7 discs a column volume of ± 69 ml. The 7.5% column volume of a 5 cm Ø disk column thus increased 12.5 fold as compared to a 1 cm Ø column and this was accompanied with an increase of ± 244 μmol to ± 3020 μmol epoxy respectively. A similar increase is observed for 6% columns with values from ± 200 μmol to ± 2560 μmol epoxy for respectively a 1 cm Ø and a 5 cm Ø column. Increase of available epoxy content creates higher binding capacity for the phage ligand and ultimately a higher binding capacity for the target of interest resulting in a higher yield.

For further evaluation of the cryogel disk columns we worked with 7.5% 2.6 cm Ø disk columns with an epoxy content of ± 2000 μmol / column (14 stacked discs). 5 cm Ø columns have indeed a higher epoxy content, but are harder to handle and to stack.

Protein binding experiments

Influence of linear velocity

Due to the high porosity of the cryogel, high linear velocities up to 750 cm/h can be applied to the column with very low flow resistance [20,21,22]. However as the interaction between the phage ligand and the target can be categorized as a “pseudoaffinity” interaction, the target should stay long enough in the column to ensure good binding. Too high linear velocities thus should be avoided to achieve optimum binding conditions. We applied linear velocities between ± 5.5 cm/h and ± 35 cm/h (Fig. 4). A decrease of $\pm 10\%$ in target binding capacity was observed when the linear velocity was increased up to 35 cm/h as compared to 5.5 cm/h. For further experiments we set a fixed linear velocity of 22.5 cm/h without losing substantial binding capacity.

Binding experiments

2.6 cm Ø 14 Disc columns were evaluated for binding capacity by varying the amount of phage ligand with or without introducing spacers. Phages were bound on the column at 1×10^{10} , 1×10^{11} and 1×10^{12} phage/ml in 65 ml phage solution. Results show (fig. 5) that there is no marked increase in HuLF binding capacity when the phage concentration is increased from 1×10^{10} up to 1×10^{12} phages/ml in a column without

spacer.

The use of spacers in between the column and the phage increases the final yield of target binding (Noppe et al., 2017). We have prepared a 2.6 cm Ø 14 disc column with a 1,6 diaminohehexane (1,6 dAH) or Lupasol FG spacer followed by coupling 65 ml phage solution (1×10^{12} phages/ml) similar to the columns described above. And indeed when running the same volume of milk sample on these stacked disc columns we observed an additional 2-10 fold increase of binding capacity depending on the spacer used. As the Lupasol FG spacer column showed the highest binding capacity we used this column to examine the binding saturation profile (fig. 5B). Results show that by loading a 50 ml sample we reach the saturation point of the column. For this specific column we can finally elute ± 5.5 mg of protein. In line with earlier results (Noppe et al., 2017) we found that the eluted protein has a purity of 90-95%, meaning that we have eluted ± 5 mg ($= \pm 0.7$ nmol) of pure human lactoferrin from the column. We have used 6.5×10^{13} phages for coupling. When assuming that nearly all phages are coupled to the column as there is a vast excess of available coupling groups by using the branched spacer, we can expect ± 0.55 nanomol peptide expressing p3 protein, which is defined as the ligand, available on the column. The amount of bound HuLF therefore would be in the same order as the amount of available ligand, which is in accordance with the experimental result showing column saturation in this set-up.

Conclusion

By upscaling from a 1 cm Ø non-spacer Hu5 phage column to a 2.6 cm Ø 14 disc-LupasolFG-HuN phage column we observed a 70 fold increase in the binding yield by optimizing several column parameters such as choice of phage ligand, phage concentration for coupling, choice of spacer, sample volume and column volume. In order to increase the binding yield, we will further optimize the production of larger disc columns and more specific coupling procedures for the phage ligand.

Also, to the best of our knowledge, so far we are the first to introduce cryogels as chromatographic matrix in combination with peptide-expressing phages as affinity ligand in a chromatographic platform. In this paper we further optimized the platform (increasing capacity). In the way we believe that we could significantly improve our platform and that by this it can become useful in many more applications.

Acknowledgements

BASF Antwerp is greatly acknowledged for the gift of Lupasol FG 800. The research was supported by a grant from the KU Leuven Industrial Research Fund (C32/16/006).

Conflict of interest

The authors have declared no conflict of interest.

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Legends

Fig. 1 Freezing thermograms of different column/plate formats

(A) 1 cm Ø column (red); 2.6 cm Ø column-edge (black); 2.6 cm Ø column-middle (blue); 5 cm Ø column-edge (green); 5 cm Ø column-middle (grey). (B) 1 cm Ø column (red); 0,5 cm plate mold (blue). (C) A cross-cut of a 5 cm Ø column: red arrows shows big irregular pores in the middle of the column due to uneven polymerization caused by the temperature gradient over the column during freezing.

Fig. 2 SEM pictures of disc cryogels

SEM pictures taken from above and the side of a cryogel discs according to the attached profile of the pictures show a comparable pore structure. The stereo picture gives an idea of the tridimensional structure of the cryogel (can be seen by using stereo glasses). Below are pictures of different formats of cryogels: below left: 1 cm and 1.6 cm column format; below right: 1 cm; 2.6 cm and 5 cm Ø cryogel discs.

Fig. 3 Epoxy concentration in cryogel discs

[epoxy] in cryogels is measured by Cu^{2+} -binding of different column/discs formats. [epoxy] is correlated to the column volume and % of monomer in the cryogel. A 6% monomer column (black) and a 7.5% column (red). The epoxy amount is expressed as $\mu\text{mol epoxy} \pm \text{SEM}$ (n=3)

Fig. 4 Amount of bound protein at different flow rates.

On a HuL phage 2.6 cm Ø disk column (14 discs), two ml of human milk sample was

applied at different flow rates. Bound protein was recovered and measured based on the Folin-Ciocalteus method. The amount of protein is expressed in μg bound protein $\pm$ SEM (n=3).

Fig. 5 Binding capacity of phage column

(A) Similar 2.6 cm $\varnothing$ epoxy-phage disc columns (14 discs) were prepared by coupling different amounts of HuN phage clone with or without spacer. All columns were run at 2 ml/min and different volumes of human milk sample are applied. 2 ml (yellow bar); 5 ml (blue bar); 10 ml (red bar) and 50 ml (green bar) of human milk sample were applied. The spacers used are Lupasol FG (100a, branched spacer) and 1,6 diaminohehexane (100b, linear spacer). The amount of protein bound is expressed as μg bound protein (mean, n=3). (B) Saturation profile of bound protein on a Lupasol FG –HuN disc column (100a) in function of the applied sample volume (mean $\pm$ SEM, n=3).

Table 1 Chromatographic running parameters

All samples are run in three fold on Äkta Pure (GE Healthcare) under similar conditions as shown in table1.

Figure 1

Figure 2

Figure 3

Figure 4.

Figure 5.

Table 1: Äkta running parameters

STEP	Flow rate (ml/min)	Time (min)	Sample Volume (ml)	%A	%B	Fraction size (min)
Column Equilibration	2	5	-	100	0	-
Sample application	2		2 / 5 /10/ 50	100	0	-
Column wash	2	45	-	100	0	-
Elution	2	40	-	0	100	1
Column wash	2	60	-	100	0	-