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Characterization of supermacroporous monolithic polyacrylamide based matrices designed for chromatography of bioparticles

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Abstract

Supermacroporous monolithic acrylamide (AAm)-based cryogels were prepared by radical cryo-polymerizaton (polymerization in the moderately frozen system) of AAm with functional monomers and cross-linker *N*,*N*′-methylene-bis-acrylamide (MBAAm). Electron microscopy studies revealed supermacroporous structure of the developed cryogels with pore size of 5–100 μm. Cryogel porosity depended on cryo-polymerization conditions. More than 90% of the monolithic bed volume is the interconnected supermacropores filled with water and less than 10% of the monolithic volume is pore walls. The total protein binding capacity (lysozyme in the case of immobilized metal affinity chromatography (IMAC) column and bovine serum albumin (BSA) in the case of anion-exchange (AE) column) was independent of the flow rates till 600 cm/h. Chromatographic behavior of *E. coli* cells when a cell suspension was applied to ion-exchange cryogel columns depended on both the density of functional ligand and the porosity of the cryogel.

Keywords: Monolithic column; Bioparticles; Cryogel; Supermacroporous polyacrylamide

1. Introduction

There is an increasing demand for highly purified preparations of bioparticles like viruses, cell organelles and viable populations of specific cells. Chromatography of cells based on the specific properties of cell surfaces has been identified as a suitable process for cell separation [1].

Traditional packed-bed chromatography with immobile stationary phase, however, despite its elegance and high resolving power, suffers from a main drawback—incapability of processing particulate-containing fluids so it is poorly suitable for processing cell suspensions. Cells are trapped in between the beads of the chromatographic carrier resulting in increased flow resistance of the column and finally complete blockage of the flow. To circumvent this drawback, an expanded-bed chromatography has been proposed [2]. However, despite all its advantages, expanded bed chromatography requires special type of columns and equipment and cannot be fitted in traditional packed-bed chromatographic systems.

Recently we have introduced continuous chromatographic columns with pores large enough to accommodate cell debris and even the whole cells without being blocked [3,4]. The supermacroporous monolithic cryogels have been produced by radical co-polymerization of acrylamide (AAm) with allyl glycidyl ether (AGE) or 2-(dimethylamino)ethyl methacrylate (DMAEMA) and cross-linker *N*,*N*′-methylene-bis-acrylamide (MBAAm) in moderately frozen systems. The ice crystals formed after freezing performed as porogen, while the dissolved monomers and initiator were concentrated in a small fraction of a non-frozen fluid in which polymerization proceeded efficiently despite that the whole system looked like a frozen ice block. After melting, a continuous gel (cryogel from the Greek κριοσ (kryos) meaning frost or ice) was formed. The gel has a sponge-like morphology and pore size of 5–100 μm. When Cu^{2+}-iminodiacetate (Cu^{2+}-IDA) ligands were introduced via AGE modification with IDA, the continuous columns were successfully used for the direct capture of His-tagged recombinant lactate dehydrogenase (His_6-LDH)

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from crude cell homogenate [3]. Cu^{2+}-IDA-cryogels and anion-exchange (AE) (produced by incorporation of DMAEMA instead of AGE into the polymerization feed) were used for chromatography of viable *E. coli* cells [4].

The porous structure of the monolithic cryogels depended on the concentration of monomers and the content of cross-linker (MBAAm) in the reaction feed and the freezing conditions [3]. The paper presents further characterization of the supermacroporous polyacrylamide based monolithic cryogels developed for the chromatography of bioparticles. The effect of freezing temperature on the properties of anion-exchange cryogels was investigated for monolithic cryogels prepared in plastic syringes (5 ml) at −12 and −18 °C.

2. Materials and methods

2.1. Materials

Acrylamide (AAm, more than 99.9% purity, electrophoresis reagent), *N*,*N*,*N*′,*N*′-tetra-methyl-ethylenediamine (TEMED) and ammonium persulfate (APS) were from BioRad (Hercules, CA, USA), MBAAm was from Acros (Geel, Belgium); DMAEMA, AGE (99%) and ethylenediamine (99%) were from Aldrich (Aldrich, Steinheim, FRG). Lysozyme (from chicken egg white) (Lyz), Cibacron Blue 3GA (C. Blue), copper sulphate, EDTA–tetrasodium salt and imidazole were from Sigma (St. Louis, USA). IDA was from Fluka (Buchs, Switzerland). Albumin fraction V from bovine serum albumin (BSA) and sodium chloride were from Merck (Darmstadt, Germany); *N*-(2-hydroxethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) was obtained from BDH Laboratory Supplies (Poole, England). Beaker's yeast were purchased in the form of pressed blocks from a local supplier. High salt LB-Broth was obtained from Duchefa (Haarlem, The Netherlands).

2.2. Methods

The production of supermacroporous columns bearing IDA-ligands and the quantification of ligand density were described in detail elsewhere [3]. The monolithic cryogels with functional epoxy groups were dried (oven at 60 °C overnight) and stored in dried state at room temperature until required. The cryogels were swollen in water and re-equilibrated in running buffer prior to chromatographic experiments.

Co-polymerization of AAm with DMAEMA and MBAAm was performed according to [4]. The anion-exchange cryogels were dried in oven at 60 °C overnight and stored in dry state until required. Tertiary amino groups in anion-exchange cryogel were titrated according to [4].

2.2.1. Characterization of continuous supermacroporous cryogel matrices

The degree of swelling of the gels ($S_{w/w}$) was determined as described elsewhere [3]. The flow rate of water passing through the columns was measured at the constant hydrostatic pressure equal to 100 cm of water-column corresponding to a pressure of circa 0.01 MPa. For each sample the average of at least three measurements was taken.

The flow resistance of the cryogels columns (5 ml) was evaluated at flow rates of 1–15 ml/min (peristaltic pump Alitea XV) registering the flow rate at given pump settings. In a separate experiment, the pump settings were calibrated against flow rate with no column connected according to [5]. The results are presented as measured flow rate versus the set-up flow rate (according to the pump settings). The flow resistance of 5 ml columns packed with Sephacryl SF-1000 and Sephadex G-100, respectively, was measured for comparison.

The water content of cryogels was estimated as follows: the swollen monolithic cryogel matrix (5 ml) was dried in an oven (60 °C) till constant weight and weight of the dried polymer sample was determined ($m_{\text{dry polymer}}$). Then the dried sample was placed into a water vapor saturated chamber with no direct contact of the sample with water. The increase in sample weight with time due to absorbed water vapor was checked for 7 days. This value gave the weight of the gel matrix with polymer bound water ($m_{\text{dry polymer+ bound water}}$). The content of dry polymer and polymer with bound water in the swollen gel was determined as percent of swollen gel weight ($m_{\text{swollen gel}}$). The total volume of supermacropores in the swollen cryogel was roughly estimated as follows: the weight of the sample ($m_{\text{squeezed gel}}$) was determined after squeezing the free water from the swollen gel matrix, the porosity was calculated as follows: $(m_{\text{swollen gel}} - m_{\text{squeezed gel}})/m_{\text{swollen gel}} \times 100\%$.

HETP values for monolithic cryogels were determined using acetone (molecular weight, MW 58 Da), BSA (MW 69 kDa), Blue Dextran (MW 2000 kDa) and *E. coli* cells as markers. Monolithic cryogels were removed from 5 ml syringes and packed into a chromatographic column (10 mm i.d.) equipped with upper and lower adapters and connected to a Biologic HR Chromatographic system (BioRad, Hercules, CA, USA). All markers were applied in 20 mM Tris–HCl buffer, pH 7.0 containing 0.5 M NaCl. Chromatographic peaks were recorded at 280 nm after injecting 50 μl marker solution (or cell suspension, OD_{600} 0.42) at flow rates of 0.1–10 ml/min. Total plate number (N) was calculated according to the formula:

$$N = 5.55 \times \left(\frac{t_R}{w_{1/2}}\right)^2,$$

where t_R is retention time of the marker and $w_{1/2}$ is the width of a chromatographic peak at half of its height. HETP values were calculated by dividing N by column height.

2.2.2. Preparation of cryogel samples for SEM

The cryogel samples for scanning electron microscopy (SEM) were fixed in 2.5% glutaraldehyde in 0.12 M sodium phosphate buffer, pH 7.2 overnight, post-fixed in 1%

osmium tetroxide for 1 h. Then the samples were dehydrated in ethanol (0–50–75–99.5%) and critical point dried [3]. The dried sample was coated with gold/palladium (40/60) and examined using a JEOL JSM-5600LV scanning electron microscope.

2.2.3. Protein chromatography

For Lyz chromatography on Cu^{2+}-IDA column 20 mM HEPES, pH 7.0 containing 0.2 M NaCl was used as running buffer and 0.2 M imidazole in the running buffer as elution buffer. After each chromatographic run, the regeneration with 0.1 M EDTA was performed for stripping Cu^{2+}-ions from the column. Then the column was washed with distilled water and loaded with Cu^{2+}-ions by passing 0.1 M $CuSO_4$ through the column. The chromatography was monitored using a LKB UVI-cord equipped with a 276 nm filter. Protein content was determined with BCA method [3].

Chromatography of BSA was carried out using 20 mM Tris–HCl buffer, pH 7.0 as a running buffer. BSA solution (0.1 mg/ml) was applied to anion exchange cryogel column (5 ml) followed by washing with running buffer until 276 nm readout was down to baseline. Elution was performed with 1.5 M NaCl in running buffer. Fractions of 3 ml were collected and optical density at 280 nm was measured. BSA content was calculated using calibration curve obtained for BSA solutions of known concentration (0.1–1 mg/ml) at 280 nm.

2.2.4. Cell chromatography

Yeast cell feedstock was prepared as follows: cell suspension (20 mg/ml suspension) in 0.15 M NaCl solution was heated for 40 min at 70 °C. Then cells were washed twice by centrifugation (5000 × *g*, 15 min) and re-suspending in 20 mM Tris–HCl buffer, pH 7.0. Cell suspension with turbidity about 1.0 at 600 nm was used for chromatography. Yeast chromatography was carried out in 20 mM Tris–HCl buffer, pH 7.0 containing 0.01 or 1 M NaCl.

E. coli cell feedstock was prepared as follows: *E. coli* cells were cultivated on agar plate over night at 37 °C. Single colonies were transferred to individual test tubes containing 10 ml LB-medium and were cultivated at 37 °C in a shaking incubator at 200 rpm over night. The tubes were used for inoculating 200 ml LB-medium. The cultivation in the flask was carried out at 37 °C in a shaking incubator at 120 rpm. After 3 h, cells (OD_{600} of 1.5) were harvested by centrifugation at 6000 × *g* for 5 min. The cell pellet (2 g) was re-suspended in running buffer (20 mM HEPES with 50 mM NaCl, pH 7.0). Cell suspension with turbidity about 1.0 at 600 nm was used for chromatography.

E. coli cell chromatography on anion-exchange monolithic columns (5 ml) was carried out using 20 mM HEPES buffer, pH 7.0 with 50 mM NaCl as a running buffer. Elution was performed stepwise with 0.2 and 1.0 M NaCl in running buffer or with a linear salt gradient 0.05–1.0 M NaCl in running buffer.

Chromatographic separation of a suspension of mixture of *E. coli* and yeast cells was performed using anion-exchange cryogel column composed of two monolithic cryogels, 5 ml each, prepared at −12 °C (DMAEMA/AAm 0.08 mol/mol), packed into a glass chromatographic column (12 mm i.d.) and equipped with upper and lower adapters. The chromatographic runs were performed using 20 mM Tris–HCl with 50 mM NaCl, pH 7.0 as a running buffer. Elution was performed with a linear salt gradient 0.05–1.0 M NaCl in running buffer. The summit fractions of the elution peaks were concentrated (14 times), heat treated to suppress growth according to [2] and the number of cells was determined by counting cells in a counting chamber using an microscope (Nikon Corporation, Japan).

3. Results and discussion

3.1. Structure and properties of cryogels obtained after cryo-polymerization at −12 and −18 °C

When cryogels are produced, polymerization takes place in the non-frozen fluids containing concentrated dissolved monomers and initiator. The ice crystals formed during freezing perform as porogen (Fig. 1). Thus, the shape and size of the crystals formed determine the shape and size of the pores formed after defrosting the sample. In general, the size of ice crystals depends on how fast the system is frozen, provided other parameters (e.g. concentration of the dissolved substances, volume and geometrical shape of the sample) remain the same [6]. The freezing rate is determined by the starting temperature, which was maintained in our case close to 0 °C as all the solutions before mixing were kept in an ice bath, and the freezing temperature, which was −12 or −18 °C, respectively. In order to obtain a reproducible freezing pattern, several precautions

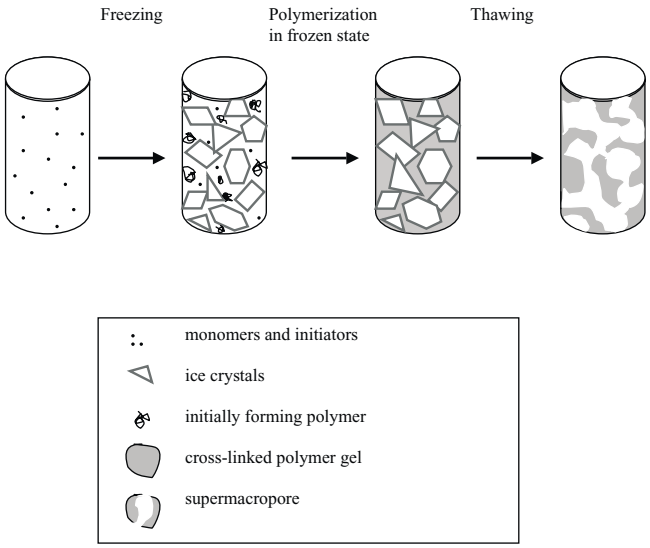
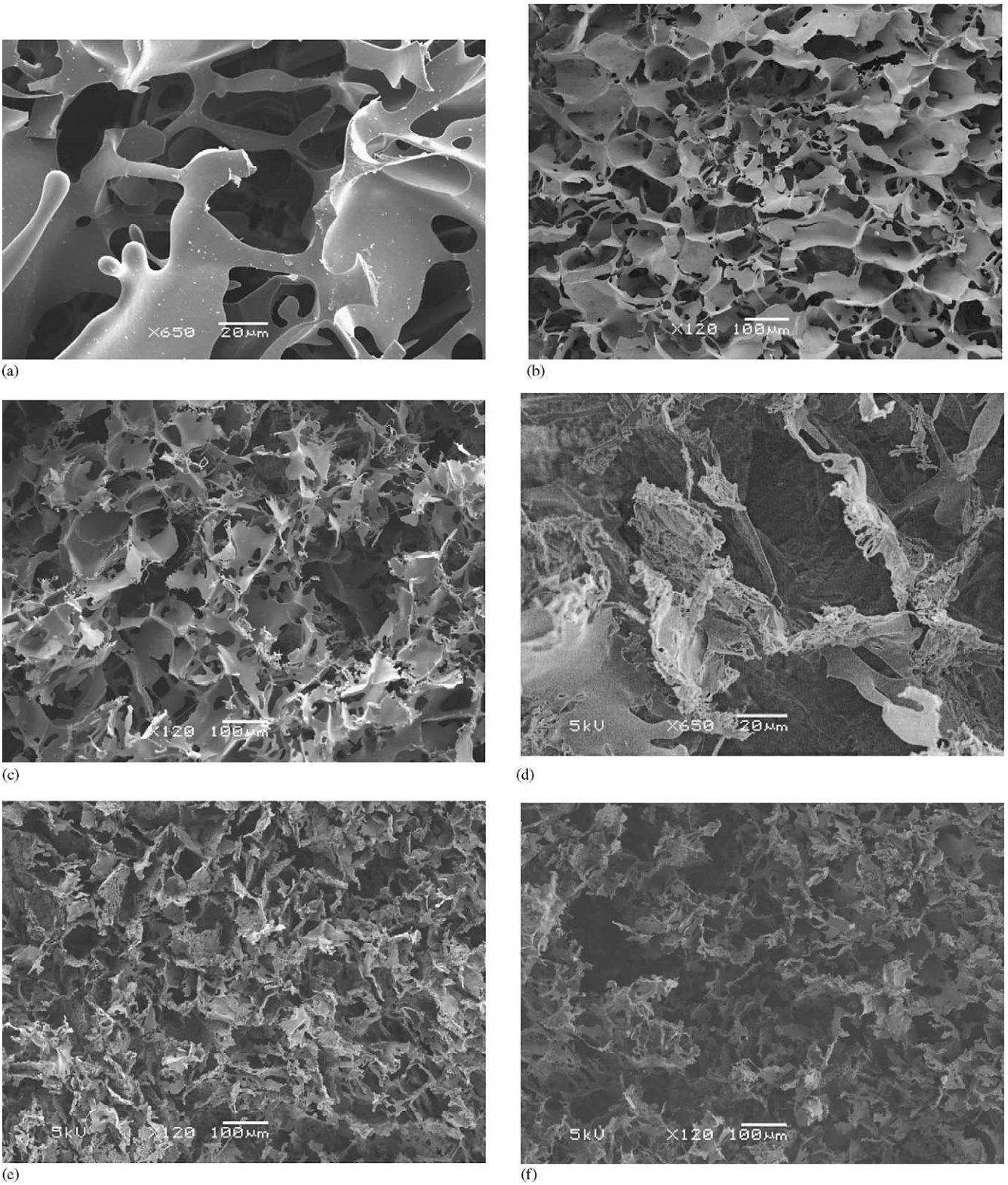


Fig. 1. Scheme of cryogel production.



(a) (b) (c) (d) (e) (f)

Fig. 2. SEM microphotograph of diametrical (a, b, d and e) and longitudinal (c and f) cross-sections of anion-exchange cryogels prepared at −12 and −18 °C, respectively. The samples were fixed in 2.5% glutaraldehyde in 0.12 M sodium phosphate buffer, pH 7.2 overnight, post-fixed in 1% osmium tetroxide for 1 h, dehydrated in ethanol and critical point dried (see Section 2.2). The dried sample was coated with gold/palladium (40/60) and examined using a JEOL JSM-5600LV scanning electron microscope.

were made: the samples of small size and uniform shape (5 ml syringes filled with 5 ml solution) were used and the time was accurately controlled from the moment when the monomer and initiator solutions were mixed (the start of polymerization reaction) to the moment when the plastic syringes filled with polymerization solution were submerged into the cooling liquid of the cryostate.

Different morphology of ice crystals formed at −12 or −18 °C, respectively, is clearly reflected in the different morphology of pores in the resulting cryogel samples (Fig. 2). The cryogels prepared at −12 °C have large (5–100 μm) interconnected pores (supermacropores) separated with smooth, nonporous dense gel walls (Fig. 2a). The cryogels prepared at −18 °C have different pore structure with an apparent bimodal pore size distribution. There are supermacropores with hundred-micrometer size and small pores about 0.1–3 μm in size (Fig. 2d). The difference in pore structure of the cryogels prepared at −12 or −18 °C, respectively, is a result of different cooling rate. At −18 °C, the samples appeared to be frozen within 4–6 min, while it took 12–15 min at −12 °C.

The different pore morphology resulted in different flow resistance of the columns filled with cryogels produced at −12 or −18 °C, respectively (estimated as water flow rate through the monolithic cryogel at hydrostatic pressure equal to 1 m of water column). The flow rate of water through the cryogel column prepared at −12 °C, was 750 cm/h, whereas different pore morphology in cryogel prepared at −18 °C resulted in a drastic decrease of the flow rate to 230 cm/h.

Flow rates as high as 750 cm/h at minimal pressure drop indicate that supermacroporous structure of cryogels endures columns packed with cryogels with very low flow resistance. Indeed, for the columns of the same geometry packed with Sephadex G-100 (Fig. 3, closed circles) and Sephacryl S-1000 SF (Fig. 3, closed squares) the back pressure (deviation from the linearity) was already noticeable at pumping speeds of about 3 and 8 ml/min. In contrast, for the columns packed with AGE-cryogel or ion-exchange cryogel no deviation from linearity was observed up to flow rates of 14 ml/min.

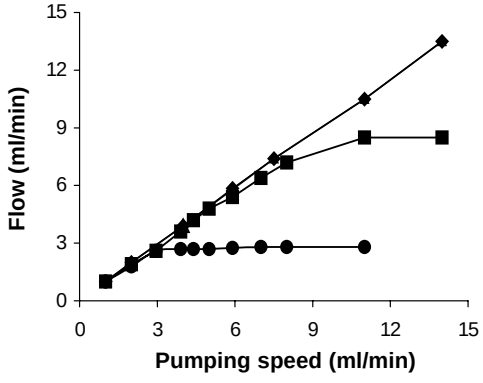


Fig. 3. Flow resistance of columns packed with Sephadex G-100 (closed circles), Sephacryl S-1000 SF (closed squares), IDA-cryogel (open triangles) and anion-exchange cryogel (closed rhombus). The results are presented as measured flow rate vs. the set-up flow rate determined in a separate experiment when the pump settings were calibrated against flow rate with no column connected. Supermacroporous monolithic cryogels were prepared in 5 ml syringe at −12 °C.

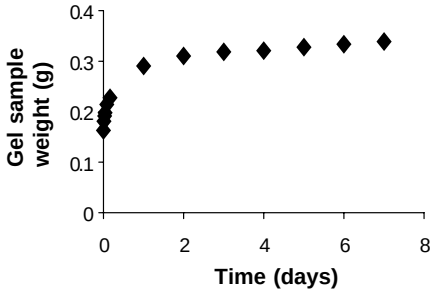


Fig. 4. Adsorption of water vapors by dry cryogel sample. AGE-cryogel (5 ml) was prepared at −12 °C and dried in an oven at 60 °C till constant weight. The dried sample was incubated in a chamber saturated with water vapors but with no direct contact with water.

Cryogels produced from polyacrylamide can be dried at 60 °C and stored nearly indefinitely in the dry state. A dry cryogel sample slowly adsorbs water vapors about 100% of its weight (Fig. 4). When submerged into liquid, dry cryogels re-hydrated within less than a minute without deterioration of their supermacroporous structure. This property of cryogels makes them very attractive as potential chromatographic carriers.

An important feature of the chromatographic material is the pore volume. On average, dry polymer constitutes only 3–4% of the total weight of completely swollen cryogel. About 4–5% of the weight is composed of water tightly bound by the polymer (adsorbed from water vapor). Together, polymer with tightly bound water form the pore walls. Thus, the remaining nearly 90% of the cryogel weight is represented by the water inside the pores. Due to the elasticity of cryogels, a large amount of water in large pores (more than 70% for cryogels prepared at −12 °C and 50–55% for cryogels prepared at −18 °C) could be squeezed mechanically from the swollen monolithic samples. It indicates that the main part of the cryogel volume is composed of the interconnected supermacropores (Fig. 5).

3.2. Chromatographic properties of monolithic cryogel columns

Low flow resistance of cryogels due to the system of large interconnected pores endures cryogel-packed columns with interesting chromatographic properties. The transport of solutes inside the monolithic cryogel column proceeded mainly due to the convection rather than due to the diffusion. The HETP values for the monolithic column produced at −12 °C were independent on the solute size for acetone (MW 58 Da), BSA (MW 69 kDa), Blue Dextran (MW 2000 kDa) and in the wide range of flow rates 0.2–10 ml/min (Fig. 6). Even the objects of μm-large size like *E. coli* cell were

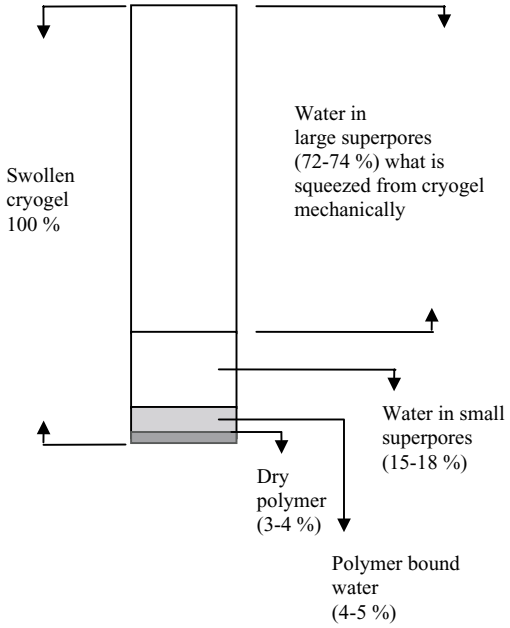


Fig. 5. Composition of the supermacroporous monolithic cryogel prepared at −12 °C. Average values are presented after study of 5 different samples. For experimental details see Section 2.

transported inside large pores of cryogel monolith with the same efficiency as soluble substances (Fig. 6, open squares).

The break-through profiles of Lyz on Cu^{2+}-IDA-cryogel column are essentially independent of the flow rate despite that the flow rate was increased an order of magnitude (Fig. 7a). The static binding capacity of Cu^{2+}-IDA-cryogel column for Lyz was 0.2 mg/ml. Low binding capacity of Cu^{2+}-IDA-cryogel with respect to the protein is due to the large pore size of the material. Increase in pore size 100–1000-fold as compared to ordinary gels which have pore size 0.03–0.4 μm [7] results in 100–1000-fold decrease in pore surface area provided the total pore volume remains

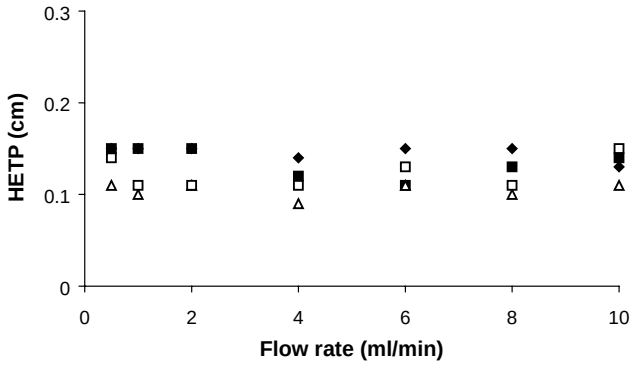


Fig. 6. The dependence of HETP on flow rate for acetone (MW 58 Da, open triangles), BSA (MW 69 kDa, closed squares), Blue Dextran (MW 2000 kDa, closed rhombus) and *E. coli* cells (open squares). Cryogel column was prepared at −12 °C. All markers were applied in 20 mM Tris–HCl buffer, pH 7.0 containing 0.5 M NaCl. Chromatographic peaks were recorded at 280 nm after injecting 50 μl marker solution (or cell suspension, OD_{600} 0.42).

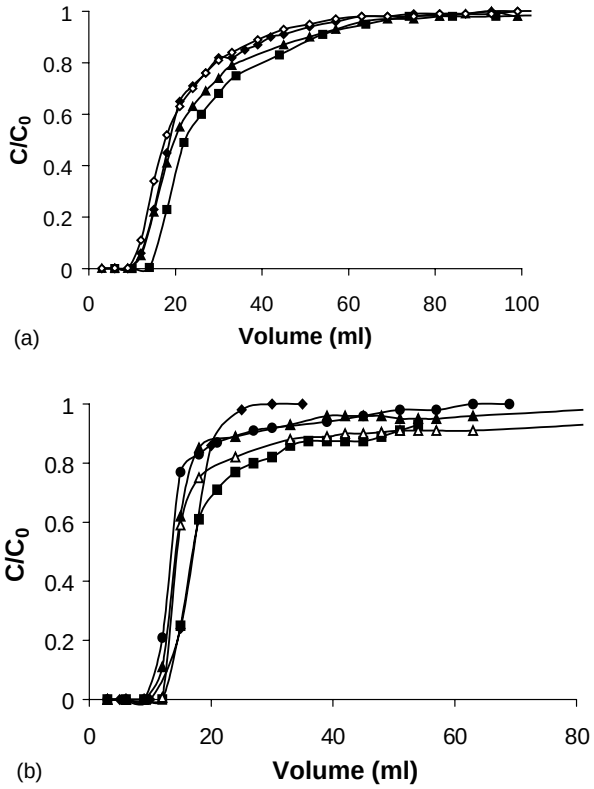


Fig. 7. Breakthrough profiles of (a) lysozyme on Cu^{2+}-IDA-cryogel columns (5 ml syringe) and (b) BSA on anion-exchange cryogel column (5 ml syringe) at different flow rates: (a) 1 ml/min (closed rhombus), 2 ml/min (closed squares), 6 ml/min (closed triangles), 12 ml/min (open rhombus). Cu^{2+}-IDA-cryogel was prepared at −12 °C. Lysozyme was applied to the column in 20 mM HEPES, pH 7.0 containing 0.2 M NaCl. (b) 0.5 ml/min (closed squares), 1 ml/min (closed rhombus), 3 ml/min (closed triangles), 6 ml/min (open triangles), 12 ml/min (closed cycles). Anion-exchange column was prepared at −12 °C using DMAEMA/AAm molar ratio 0.08. BSA was applied to the column in 20 mM Tris–HCl buffer, pH 7.0.

the same. Hence, the area available for protein binding will be drastically decreased resulting in decreased capacity of the cryogel.

Ion-exchange functionality was introduced into cryogel directly during polymerization using DMAEMA as one of co-monomers [4]. The higher the content of DMAEMA in the polymerization feed, the higher was the static capacity of the ion exchange cryogel columns. The capacity of the ion-exchanger was estimated from titration of amino groups incorporated into the cryogel (data not shown). The cryogels with good sponge like structure were obtained when molar ratios DMAEMA/AAm in the polymerization feed were in the range of 0.08–0.2. Further increase in DMAEMA content resulted in the formation of brittle rather than elastic cryogels. Total capacity of retained model protein BSA increased with increasing the content of DMAEMA in the reaction mixture (Fig. 8). Again, breakthrough profiles of BSA on the anion-exchange cryogel column were independent of the flow rate (Fig. 7b). Some compression of gel matrix (10–15% of total height) was observed at flow rates

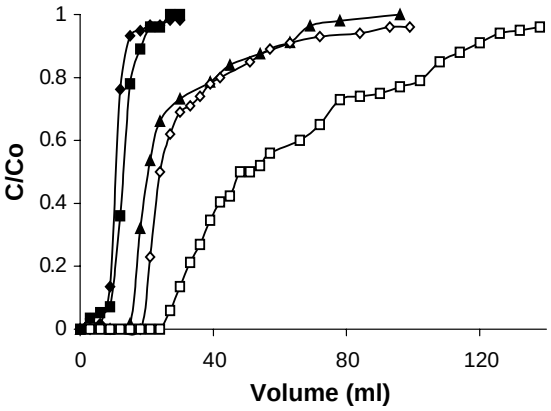


Fig. 8. Breakthrough curves of BSA on anion-exchange cryogel column (5 ml syringe) prepared at −12 °C using DMAEMA/AAm molar ratios: 0.04 (closed rhombus), 0.08 (closed squares), 0.14 (closed triangles), 0.2 (open rhombus), 0.4 (open squares).

above 12 ml/min resulting in some decrease in static capacity (Table 1) probably due to closure of some pores when the gel compression occurred.

It should be noted here that the static capacities for BSA of anion-exchange columns prepared at −12 and −18 °C, respectively, using the same content of functional monomer in the polymerization feed (DMAEMA/AAm molar ratio 0.08) were practically the same, 0.18–0.22 mg/ml, despite the clear differences in the cryogel structure (compare Fig. 2a and d).

The supermacropores in monolithic cryogels are big enough to allow microbial cells to pass freely through the monolithic supermacroporous cryogel without any blocking of the cryogel. *E. coli* cells were not retained by plain cryogel columns prepared at −12 and −18 °C. More than 90% of the cells appeared in a breakthrough fraction when the cell suspension was applied to cryogel columns. Yeast cells, which are larger in size were partially retained by the cryogel columns with 70 and 57% of yeast cells passing freely through the cryogels prepared at −12 and −18 °C, respectively.

More than 90% of *E. coli* cells were also in the breakthrough fraction when the cell suspension was applied to anion-exchange cryogel column prepared at −12 °C

Table 1
Static capacity for BSA of anion-exchange cryogel column at different flow rates

Flow rate (ml/min)	Linear velocity (cm/min)	Compressing of gel matrix (%)	Total retained BSA (mg/ml)
0.5	0.41	0	0.19
1.0	0.83	0	0.22
3.0	2.5	0	0.18
6.0	5.0	0	0.16
12.0	10.0	10–15	0.13

Anion-exchange cryogel column was prepared at −12 °C using DMAEMA/AAm molar ratio 0.08. BSA was applied to the column in 20 mM Tris–HCl buffer, pH 7.0 (running buffer) and eluted with 1.5 M NaCl in running buffer.

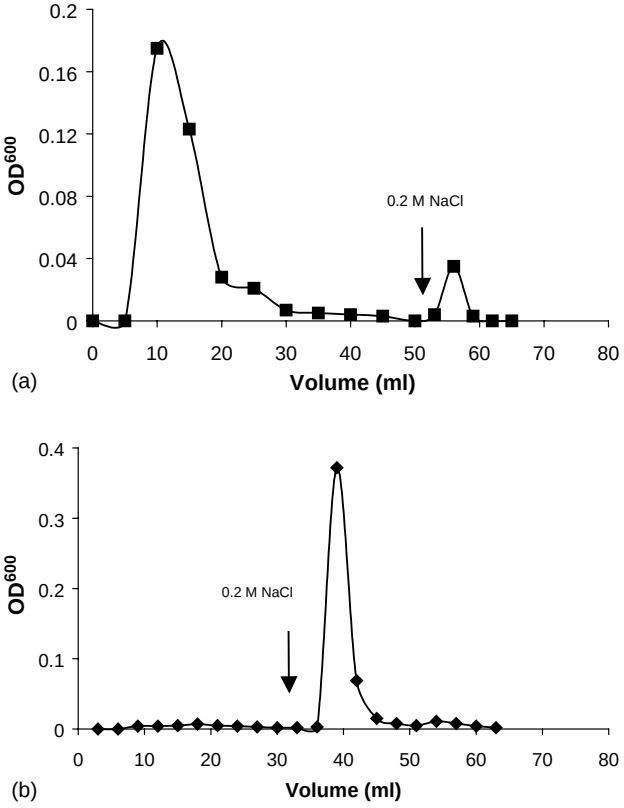


Fig. 9. Chromatography of *E. coli* cells on anion-exchange cryogel column (5 ml syringe) prepared at −12 °C with different content DMAEMA/AAm molar ratio: (a) 0.04 and (b) 0.08. Cell suspension (3 ml with OD_{600} 0.7) was applied to the columns in 20 mM HEPES buffer, pH 7.0 containing 50 mM NaCl (running buffer). Elution was performed with 0.2 M NaCl in running buffer.

with low content of functional DMAEMA co-monomer in polymerization feed (DMAEMA/AAm molar ratio 0.04) (Fig. 9a). However almost all *E. coli* cells were bound to anion-exchange cryogel column prepared at the same temperature but with higher content of DMAEMA in the polymerization feed (DMAEMA/AAm molar ratio 0.08) (Fig. 9b). Bound cells were eluted with 0.2 M NaCl in running buffer. Further increase in salt concentration till 1 M did not result in any noticeable additional elution of cells.

The difference in pore morphology of the anion exchange cryogel columns prepared at −12 and −18 °C using the same DMAEMA/AAm molar ratio of 0.08 resulted in somewhat different elution profiles when eluting bound *E. coli* cells (Fig. 10). Bound cells were eluted at lower NaCl concentration and as a broader peak from the column prepared at −18 °C as compared to the column prepared at −12 °C.

The differences in pore structure had even more pronounced effect on binding of yeast cells which are about 10-fold larger in size than *E. coli* cells. The application of a small pulse of yeast cells (4 mg in 3.3 ml) in running buffer with low salt concentration (0.01 M NaCl) to both anion-exchange cryogel columns resulted in practically

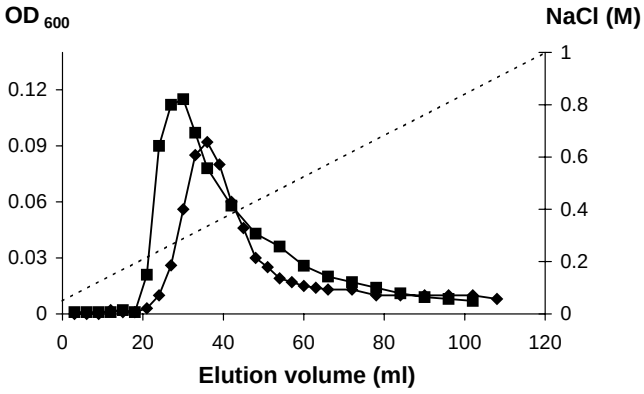


Fig. 10. Elution of *E. coli* cells from anion-exchange columns (5 ml syringe) prepared at −12 °C (closed rhombus) and −18 °C (closed squares) using DMAEMA/AAm molar ratio 0.08. The cell supension (3.6 ml with OD_{600} 1.13 and 3.1 ml with OD_{600} 1.21) was applied to anion-exchange columns prepared at −12 and −18 °C, respectively) in 20 mM HEPES buffer, pH 7.0 containing 50 mM NaCl (running buffer). Elution was performed with a linear 0.05–1.0 M NaCl gradient (120 ml) in 20 mM HEPES buffer, pH 7.0.

irreversible binding of yeast cells. No yeast cells were coming out of the column when eluting with 1 M NaCl.

The addition of high salt concentration is supposed to suppress electrostatic interaction between the negative charge on the surface of yeast cells and positive charges on the cryogel. In the presence of 1 M NaCl, more than 70% of applied yeast cells were in the breakthrough fraction for a cryogel column prepared at −12 °C but no cells were coming out for cryogels prepared at −18 °C. Obviously, the porous structure of the cryogels prepared at −18 °C with bimodal pore size distribution promoted irreversible adsorption of yeast cells even at high salt concentration.

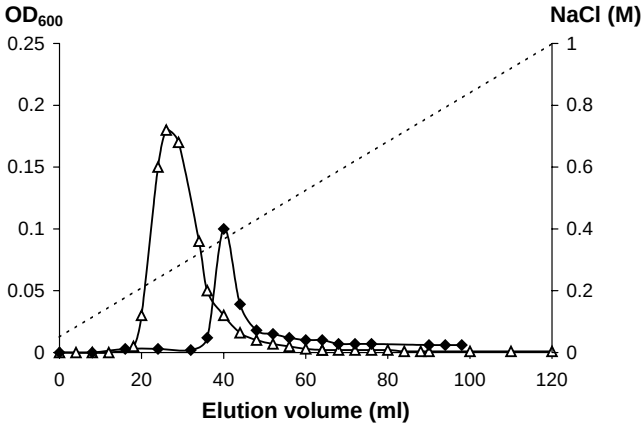


Fig. 11. Elution of bound *E. coli* and yeast cells from anion-exchange columns (5 ml) prepared at −12 °C using DMAEMA/AAm molar ratio 0.08. The cell suspension (3.4 ml with OD_{600} 1.02 for *E. coli* cells and 3.2 ml with OD_{600} 1.06 for yeast cells) was applied separately to anion-exchange columns (5 ml) in 20 mM HEPES buffer, pH 7.0 containing 50 mM NaCl (running buffer). Elution was performed with a linear 0.05–1.0 M NaCl gradient (120 ml) in 20 mM HEPES buffer, pH 7.0. Open triangles for *E. coli* cells and closed rhombus for yeast cells.

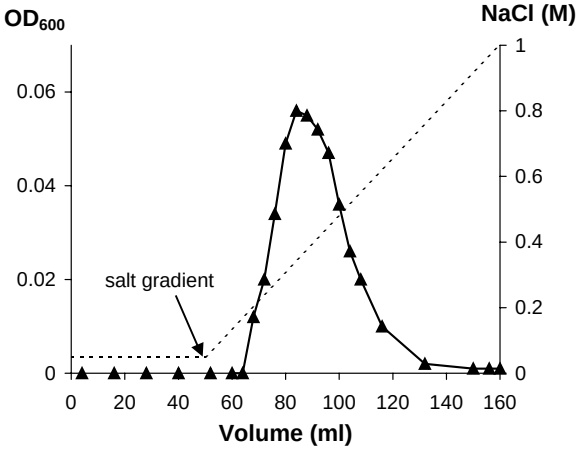


Fig. 12. Elution of bound mixture of *E. coli* and yeast cells from anion-exchange column (10 ml) prepared at −12 °C using DMAEMA/AAm molar ratio 0.08. The column was prepared by packing two monolithic anion-exchange cryogels, prepared in the plastic syringes (5 ml) at −12 °C using DMAEMA/AAm molar ratio 0.08 into a glass column (i.d. 12 mm), equipped with upper and lower adapters. Three milliliters of a mixture 1:1 (w/w) of *E. coli* and yeast cells with OD_{600} 1.0 was applied in 20 mM HEPES buffer, pH 7.0 containing 50 mM NaCl (running buffer). Elution was performed with a linear 0.05–0.0 M NaCl gradient (120 ml) in 20 mM HEPES buffer, pH 7.0.

When eluting individually bound *E. coli* and yeast cells, respectively with 0.35–0.4 M NaCl gradient, 84% of *E. coli* cells and only 32% of yeast cells were eluted from the 5 ml anion-exchange columns prepared at −12 °C (Fig. 11).Yeast cells were eluted later in the gradient at a salt concentration of 0.35–0.4 M while *E. coli* cells came out earlier at a salt concentration 0.28–0.33 M. After finding that the yeast cells bind stronger to anion-exchange cryogel than *E. coli* cells do, a mixture of *E. coli* and yeast cells (3 ml containing around 10^8 *E. coli* cells/ml and around 1.5×10^7 yeast cells/ml) was applied to the ion-exchange cryogel column (10 ml) prepared at −12 °C to test the separation performance. Elution with salt gradient resulted in a single peak (Fig. 12) with summit fractions containing only negligible amount of yeast cells. Assuming that *E. coli* cells were predominant in the elution peak (yeast cells came later), the recovery was about 60%. Thus, using monolithic ion-exchange column it was possible to achieve purification of *E. coli* cells from yeast cells from a model mixture.

4. Conclusions

The supermacroporous monolithic AAm-cryogels columns present promising separation media for isolation and purification of biological nanoparticles and even cells in chromatographic mode. The columns have very low back-pressure with more than 90% of the column volume being composed of interconnected supermacropores. The pore size and pore morphology of cryogels are controlled by the concentration of the monomers used and the freezing temperature.

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