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Chromatography of microbial cells using continuous supermacroporous affinity and ion-exchange columns

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Abstract

Continuous supermacroporous chromatographic columns with anion-exchange ligands [2-(dimethylamino)ethyl group] and immobilized metal affinity (IMA) ligands (Cu^{2+}-loaded iminodiacetic acid) have been developed allowing binding of *Escherichia coli* cells and the elution of bound cells with high recoveries. These poly(acrylamide)-based continuous supermacroporous columns have been produced by radical co-polymerization of monomers in aqueous solution frozen inside a column (cryo-polymerization). After thawing, the column contains a continuous matrix (so-called cryogel) with interconnected pores of 10–100 μm in size. The large pore size of the matrix makes it possible for *E. coli* cells to pass unhindered through a plain column containing no ligands. *E. coli* cells bound to an ion-exchange column at low ionic strength were eluted with 70–80% recovery at NaCl concentrations of 0.35–0.40 *M*, while cells bound to an IMA-column were eluted with around 80% recovery using either 10 m*M* imidazole or 20 m*M* EDTA solutions, respectively. The cells maintain their viability after the binding/elution procedure. These preliminary results indicate that microbial cells can be handled in a chromatographic mode using supermacroporous continuous columns. These columns are easy to manufacture from cheap and readily available starting materials, which make the columns suitable for single-time use.

Keywords: Cells; Stationary phases, LC; Ion-exchange chromatography; Immobilized metal affinity chromatography; Bacteria; Proteins

1. Introduction

One of the main focuses of biochemical research has been to better understand individual molecules and their roles in the living process. Great efforts have been made to obtain a better understanding on how these biomolecules are organized in more complex structures and also how these structures function in the living cell. Extensive experience of working with individual biomolecules has been

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based on the existence of highly efficient techniques for isolation and purification of molecular entities with molecular masses less than 10^6 Da. However, purification of larger objects, often combined under the name of biological nanoparticles, like plasmids, cell organelles, viruses, protein inclusion bodies, macromolecular assemblies as well as the separation of cells of different kinds, still remain a challenge. Large particle sizes (20–300 nm), low diffusion rates and complex molecular surfaces distinguish such objects from protein macromolecules (commonly <10 nm) [1].

Traditional approaches for isolation of nanoparticles, such as ultracentrifugation, micro- and ultrafiltration are limited either in scale, time or resolution. This is due to the similarities in size and density of cell debris and target nanoparticles. Alternatively, partitioning in aqueous two-phase systems (ATPS) could be used, but it suffers from the necessity to separate the target product from the phase-forming polymer [2,3]. Furthermore, extraction is a one-plate process, and one therefore needs repetitive extractions in order to obtain good resolution. This makes such a method tedious and cumbersome. When using ATPS, materials often gather in the interface and may because of this be difficult to collect.

Selective adsorption to chromatographic matrices is a method that offers many potential advantages with respect to resolution, scale-up and process integration. It is noteworthy that only a small number of commercial chromatographic matrices such as Sephacryl S-1000 SF (spherical particles from Amersham Pharmacia) are claimed to accommodate intraparticle pores up to 400 nm in diameter [1].

Nanoparticles and cells have very low diffusion coefficients due to the large size and could be forced inside the pores only by a convective flow. For beaded chromatographic matrices most of the convective flow in the column goes through the void volume between the beads. Even for recently developed supermacroporous beads with pore size of 800 nm [4,5] up to 95% of the flow passes through the void volume around the beads [6].

Svec and Fréchet [7] suggested the use of moulded continuous chromatographic media, or so-called ‘‘macroporous monoliths’’, produced by controlled polymerization inside the chromatographic column. Typically, these monoliths are produced by polymerization of styrene or acrylate monomers and contain flow-through pores with diameters in the range of 700 nm–2 μm. Later on, continuous superporous chromatographic media with pores as large as 20–200 μm were produced from agarose by Gustavsson and Larsson [8]. These pores could easily accommodate objects as large as yeast cells [9].

Continuous chromatographic media have not been used so far for selective adsorption of cells. The objective of the present study was to develop a new type of supermacroporous continuous immobilized metal affinity chromatography (IMAC) and ion-exchange chromatography (IEC) matrices and evaluate their potential as carriers for the chromatography of *E. coli* cells.

2. Materials and methods

2.1. Materials

2-(Dimethylamino)ethyl methacrylate (DMAE-MA) and allyl glycidyl ether (AGE, 99%) were from Aldrich (Steinheim, Germany). Acrylamide (AAm, more than 99.9% pure, electrophoresis reagent) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were from BioRad (Hercules, CA, USA). *N*,*N*′-methylene-bis(acrylamide) (Bis-AAm) was from Acros (Geel, Belgium). 2,4,6-trinitrobenzensulfonate (TNBS, 5% aqueous solution), sodium pyruvic acid (pyruvate), copper sulfate, EDTA-tetrasodium salt and imidazole were from Sigma (St. Louis, MO, USA). Iminodiacetic acid (IDA) was from Fluka (Buchs, Switzerland). Albumin fraction V from bovine serum (BSA) and sodium chloride were from Merck (Darmstadt, Germany). Tryptone and yeast extracts were bought from DIFCO (Detroit, MI, USA). *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) was bought from BHD Laboratory Supplies (Poole, UK). High salt LB-Broth, micro agar and sodium ampicillin was of the brand Duchefa (Haarlem, Netherlands) and bought from Saveen Biotech (Malmö, Sweden). Tris was purchased from USB (Cleveland, OH, USA).

2.2. Methods

2.2.1. Production of continuous supermacroporous IEC-columns

Continuous supermacroporous columns have been provided by Protista International (Bjuv, Sweden) and was produced as follows: Monomers (AAm + DMAEMA/MBAAm = 5.4/1, DMAEMA/AAm = 1:10) (Table 1) were dissolved in deionized water. The mixture was degassed under vacuum (water pump aspirator) for about 5 min to eliminate dissolved oxygen and pH of the solution was adjusted to 6.3–6.8 with 1 *M* HCl. Free radical polymerization was initiated by TEMED and APS. After addition of TEMED (1.0% of the total weight of monomers) the solution was cooled in an ice bath for 4–5 min. Then ammonium persulfate solution (0.8% of the total weight of monomers; cooled in an ice bath for 4–5 min) was added and the reaction mixture was stirred for 1 min. Five milliliters of the reaction mixture was poured into plastic 5-ml syringes (I.D. 12.4 mm) with closed outlet at the bottom. The solution in the syringes was frozen within 10 min at −12.0 °C. The samples were kept frozen, first at −12.0 °C for 5 h followed by storage at −15.0 °C overnight and then thawed at room temperature. The cryogel matrix prepared in each syringe was washed by passing 200 ml water at a flow-rate of 1 ml/min.

The degree of swelling ($S_{w/w}$) of the cryogels thus produced was determined as described elsewhere [10]. 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 ca. 0.01 MPa. For each column at least three measurements were done.

2.2.2. Ion-exchange capacity determination

Cryogel IEC matrix (5 ml) was dried at 60 °C till constant weight. The dry polymer matrix was cut into small pieces and transferred to a beaker containing a mixture of 40 ml 0.1 *M* HCl and 15 ml saturated NaCl solution. The mixture was kept at room temperature for 24 h at periodical agitation. It was then titrated with 0.1 *M* NaOH till pH 6.9–7.3 at slow stirring. A blank containing a mixture of 40 ml 0.1 *M* HCl and 15 ml saturated NaCl solution was titrated till the same pH value.

Titration of tertiary amino groups was performed according to Staby et al. [11]. Fifty milliliters 1.0 *M* KCl, pH 3.0 was pumped through a supermacroporous IEC column at a flow-rate of 1 ml/min. Then the cryogel IEC matrix was taken out of the column, cut into small pieces and transferred to a beaker containing 40 ml of 1.0 *M* KCl, pH 3.0. The mixture was titrated with 0.1 *M* NaOH solution up to pH 12. For comparison, DEAE Sepharose FF resin in 40 ml of 1.0 *M* KCl, pH 3.0 and a blank solution (40 ml 1.0 *M* KCl, pH 3.0) were titrated over the same pH interval.

The production and characterization of continuous supermacroporous columns bearing iminodiacetic acid ligands is described in detail elsewhere [10].

2.2.3. Cultivation of cells

Cells of *E. coli* TG1 [F^-, *tra*D36, *lac*I^q, *f*(*lac*Z)M15, *pro*A^+B^+/*sup*E, *f*(*hsd*M-*mcr*B)5, (r_k^-, m_k^--*mrc*B-), *thi*, *f*(*lac*-*pro*AB)] carrying plasmid pEVCd [12] were used. The reason for choosing ampicillin-resistant cells was to keep the analytical stages free from infection. Samples from −80 °C glycerol stock of cells were cultivated on agar plates with ampicillin overnight at 37 °C. Single colonies

Table 1
Properties of anion-exchange superporous continuous adsorbent: (AAm + DEAEMA/MBAAm = 5.4:1, DMAEMA/AAm = 1:10, mol/mol)

Total initial concentration of co-monomers (%)	Swelling degree (g water/g dry polymer)	Water flow-rate (hydrostatic pressure circa 0.01 MPa) (cm/h)	Ion-exchange capacity (μmol tertiary amino groups/g dry polymer)
5.0	5.9	720±65	1010±150
6.0	5.5	750±80	1080±250
7.0	4.3	540±64	930±280

Conditions of cryopolymerization: freezing at −12 °C for 5 h, storage at −15 °C overnight, thawing at room temperature

were transferred to individual test tubes containing 10 ml LB-medium (tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l), 100 mg/l ampicillin and were cultivated at 37 °C in a shaking incubator at 200 rpm overnight. The inoculant produced was used for inoculating 200 ml LB-medium containing 100 mg/l ampicillin in a 1 l shaking flask. The cultivation in the flask was carried out at 37 °C in a shaking incubator at 120 rpm. After 5.5 h, cells were harvested by centrifugation at 5800 *g* for 5 min. The cell pellet was kept on ice and resuspended in running buffer prior to chromatography.

2.2.4. IMAC of cells

All cell suspensions for IMAC were filtered through a 1 cm column with plain AAm/Bis-AAm continuous supermacroporous matrix to remove cell aggregates prior to chromatography. Before filtration, the cell suspensions were diluted to an A_{450} of about 1.5 and after filtration the A_{450} was adjusted to approximately 1.0. The chosen wavelength was because Cu^{2+}/EDTA solution does not absorb light at 450 nm. As running and dilution buffer 20 m*M* HEPES, 200 m*M* NaCl pH 7.0 was used. In all experiments flow-rate was 1 ml/min. The chromatography processes were monitored using a LKB UVI-cord equipped with a 276 nm filter.

Four milliliters of filtered cell suspension was applied to a 5 ml Cu^{2+}-IDA-cryogel column followed by washing with running buffer until readout was down to baseline. Elution buffer was either 10 m*M* imidazole in 20 m*M* HEPES, 200 m*M* NaCl pH 7.0 or 20 m*M* EDTA, 50 m*M* NaCl pH 8.0. The latter stripped the carrier from Cu^{2+} and was also used as the first regeneration step when elution was performed with imidazole. Regeneration then proceeded by washing the columns with distilled water (20 ml) and new Cu^{2+} was loaded by passing 20 ml 0.5 *M* $CuSO_4$ (dissolved in distilled water) through the supermacroporous column and finally washing with another 20 ml distilled water.

2.2.5. IEC of BSA

Chromatography of BSA was carried out using 10 m*M* Tris–HCl buffer, pH 7.0 as running buffer at a flow-rate of 1 ml/min. BSA solution (25 ml, 0.1 mg/ml) was applied to a 5 ml anion-exchange column followed by washing with running buffer until the 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.

2.2.6. IEC of cells

Cell chromatography was carried out using 20 m*M* HEPES, 50 m*M* NaCl, pH 7.0 as running and dilution buffer. Flow-rate was 1 ml/min. All cell suspensions were filtered through a 1 cm column with plain AAm/Bis-AAm continuous supermacroporous matrix to remove cell aggregates prior to chromatography. Four milliliters of *E. coli* suspension (A_{600} approximately 1.0) was applied to a 5 ml anion-exchange column followed by washing with running buffer. Elution was performed using a linear salt gradient 0.05–1.0 *M* NaCl (120 ml) in running buffer. Fractions of 3 ml were collected and A_{600} was measured.

2.2.7. Preparation of cryogel samples for microscopy

The samples were fixed in 2.5% glutaraldehyde in 0.15 *M* sodium cacodylate buffer pH 7.2 overnight, then postfixed in 1% osmium tetroxide for 1 h. Then samples were dehydrated stepwise in ethanol (0→50%→75%→96%→99.5%, each concentration 2 times for 10 min and kept at 99.5% overnight) and transferred to a critical point drier temperatured to +10 °C where the ethanol was changed for liquid carbon dioxide as transitional fluid. The temperature was then raised to +40 °C and the pressure to ca. 100 bar, i.e. beyond the critical pressure and temperature (+31 °C and 73 bar, respectively). Liquid CO_2 was then transformed directly to gas uniformly throughout the whole sample without heat of vaporization or surface tension forces causing damage [13]. Release of the pressure at constant temperature of +40 °C resulted in dried samples. These were then coated with gold/palladium (40/60) and examined using a JEOL JSM-5600LV scanning electron microscope.

3. Results and discussion

3.1. Production and characterization of continuous supermacroporous cryogel

Supermacroporous continuous chromatographic matrices have been produced by a direct cryo-co-polymerization (from the Greek *kryos* meaning ''frost'' or ''ice'') of monomers AAm/AGE or AAm/DMAEMA, respectively, and a cross-linking agent, Bis-AAm directly in a column. The salient feature of cryo-polymerization is crystallization of the solvent. When the temperature is decreased, the freezing of the initial aqueous solution first results in crystallization of pure water and concentration of dissolved substances in the non-frozen solvent (so-called ''cryo-concentration''). Even when the entire reaction mixture looks like a solid frozen block part of the solution with concentrated reactants remains non-frozen (0.1–10% of the total mass depending on the conditions [14]). The chemical reactions proceed in this non-frozen part of the reaction mixture. As the volume of the unfrozen liquid microphase is significantly less than the volume of initial solution, pronounced concentration of the dissolved substances takes place.

Non-frozen solution forms a continuous phase in between ice crystals. The latter perform as pore-forming agent or porogen when the process in question is a gel formation. Melting of these ice crystals results in cavities in the synthesized polymer material (cryogel). These cavities form a continuous interconnected system of pores filled with water. Surface tension at the interface of the cryogel and the liquid causes the shape of the initially sharply angled cavities to become rounded. The size of the pores and the thickness of polymer walls in between the pores are determined by the size of the ice crystals and the ratio between the non-frozen solution and the frozen solvent. Thus the morphology of cryogel can be controlled by: (1) the concentration of monomers and cross-linker in initial solution, (2) the freezing rate (controls the size of ice crystals) and (3) the temperature at which the frozen reaction mixture is stored (controls the ratio of [non-frozen solution]/[frozen solvent]) [14].

To be useful as chromatographic matrices, porous materials like supermacroporous cryogels should have some functional moieties (ligands) suitable for specific binding of target objects. In this work, we have used two types of ligands, ion-exchange (IE) ligands, comprised of tertiary amino groups which are positively charged at neutral conditions, and immobilized metal-affinity (IMA) ligands, iminodiacetic acid loaded with Cu^{2+}-ions, capable of binding imidazole moieties.

Mechanical properties of the adsorbents depend on the total concentration of co-monomer in the initial mixture. Adsorbents with good sponge-like structure and high flow-rate of water (hydrostatic pressure about 0.01 MPa) through the column were obtained from 5 to 6 w/v% of monomer solution (Table 1). For further studies adsorbents prepared from 6 w/v% of monomer solution were selected.

Measuring flow-rate through the supermacroporous continuous column is a simple way of estimating porosity. Due to the large pore size and interconnected pore-structure, the continuous chromatographic poly(acrylamide) matrix has a very low flow resistance. Water (hydrostatic pressure ca. 0.01 MPa) flows freely, 700–800 cm/h, through the 4 cm high column at a flow-rate about 18–21 ml/min (six batches with 14 columns prepared in each batch, Fig. 1). No visible changes in the properties of the continuous matrix were observed at this flow.

It is worthwhile to point out for comparison that

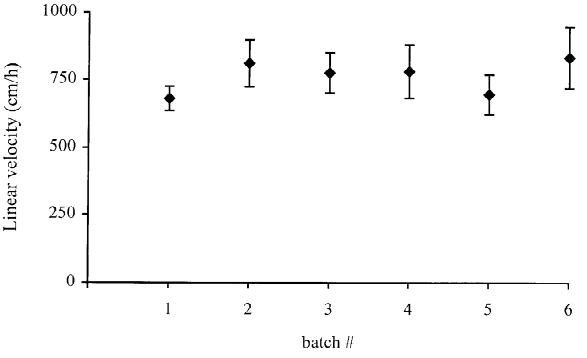

Fig. 1. Water flow-rate (hydrostatic pressure ca. 0.01 MPa) at room temperature through the supermacroporous continuous anion-exchange columns from six different batches. Fourteen columns were prepared in a single batch and six columns from each batch were randomly chosen for the estimation of water flow-rate (mean value of water flow-rate±SD is presented).

high-performance liquid chromatography (HPLC) operates at flow-rates of 300–1700 cm/h at excessive pressures of 2–10 MPa [16] and expanded bed chromatography—at flow-rates of 200–400 cm/h at pressure about 0.01 MPa [17]. Thus, it is reasonable to foresee that after an appropriate optimization, continuous supermacroporous matrices produced by the method of cryotropic gelation would allow chromatographic processes at flow-rates comparable with those in HPLC and exceeding those used in expanded bed chromatography, while using only minimal pressures typical for low pressure protein chromatography. Flow-rates up to 2000 cm/min were reported for the continuous matrices based on the copolymer of glycidyl methacrylate and ethylene dimethacrylate, so-called CIM disks (BIA Separation, Slovenia) [18]. However, these disks are only about 2 mm thick [19].

3.2. IEC cryogels

Ion-exchange functionality is introduced directly during polymerization using DMAEMA as one of the co-monomers. DMAEMA, possessing a tertiary amino group, was selected as ionogenic co-monomer to incorporate the functional dimethylamino group during polymerization.

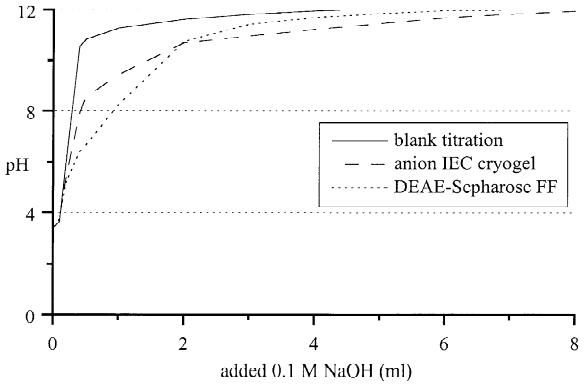

Fig. 2. Titration curve of weak anion-exchangers suspended in 1.0 *M* KCl from pH 3 to pH 12 with 0.1 *M* NaOH: solid line—blank titration in salt solution; broken line—5.4 ml supermacroporous continuous anion-exchange adsorbent (6% total initial monomer concentration), corresponding to 0.20 g dried matrix; dotted line—2 ml settled DEAE–Sepharose Fast Flow, corresponding to 0.167 g dried matrix.

Cryo co-polymerization of AAm with DMAEMA and MBAAm was performed directly in a column (length 45 mm, I.D. 12.4 mm). Initial pH of the co-monomer solution of (AAm + DMAEMA + MBAAm) was 10.6–10.8. Polymerization at very high pH might be accompanied by the hydrolysis of the amide groups [15]. Moreover, pH may dramati-

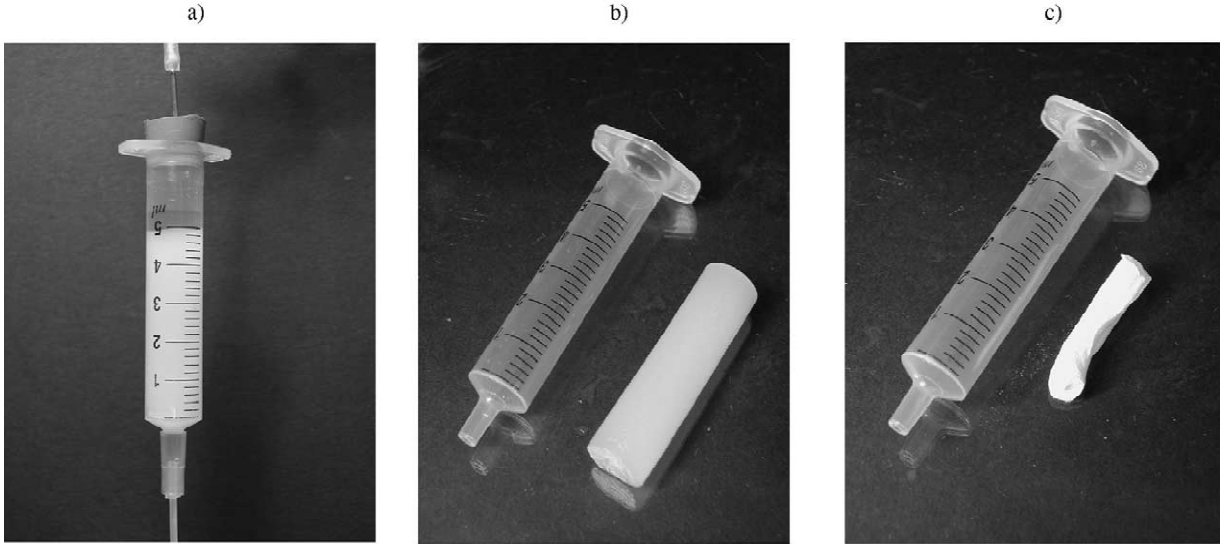

Fig. 3. (a) Continuous supermacroporous anion-exchange column. (b) Adsorbent removed from the column. (c) Dried adsorbent.

cally increase during the freezing step due to the above-mentioned cryoconcentration effect in the unfrozen liquid microphase where polymerization reaction proceeds [14]. To avoid monomer hydrolysis at high pH value during cryo-polymerization, the pH of co-monomer solution before freezing was adjusted to 6.6–6.8. The concentration of APS (0.8% of total weight of AAm+DMAEMA+MBAAm) was chosen as it produced gel at room temperature in 30–40 min. Molar ratios between the vinyl (AAm+DMAEMA) and divinyl component (MBAAm) in the range of 5:1–10:1 and DMAEMA/AAm ratio 1:10 were selected based on our previous work on producing supermacroporous continuous matrices for IMAC-purification of LDH (lactate dehydrogenase) directly from non-clarified crude homogenate [10].

The developed anion-exchange continuous supermacroporous matrix belongs to the weak type of anion-exchangers. Titration curve of DEAE Sepharose FF (commonly used weak anion-exchanger) and titration of salt solution (blank) are shown for comparison (Fig. 2).

The cryogel-based anion-exchange continuous supermacroporous adsorbent is sponge-like and elastic. The cylinder of adsorbent can be removed and put back into a column again (Fig. 3a and b). Removal is simply done by pouring out excess liquid and then with a light pressure rotate in a thin glass rod between the inside wall and the gel. Circa 4 ml of water can be squeezed out of 5 ml gel, which corresponds to the estimated flow-through pore volume [10]. The adsorbent can be dried (Fig. 3c) and rehydrated without losing its properties. Swelling degree of the polymeric phase of this heterogeneous gel material equal to 4–6 g H_2O per g dry adsorbent was close to that of continuous supermacroporous IMA-adsorbents [10]. As expected, this is less than for the traditional homophase AAm-DMAEMA-MBAAm gels (12–20 g H_2O per g of dry polymer) prepared at the same monomer/initiator/activator concentration at room temperature.

Production of supermacroporous continuous col-

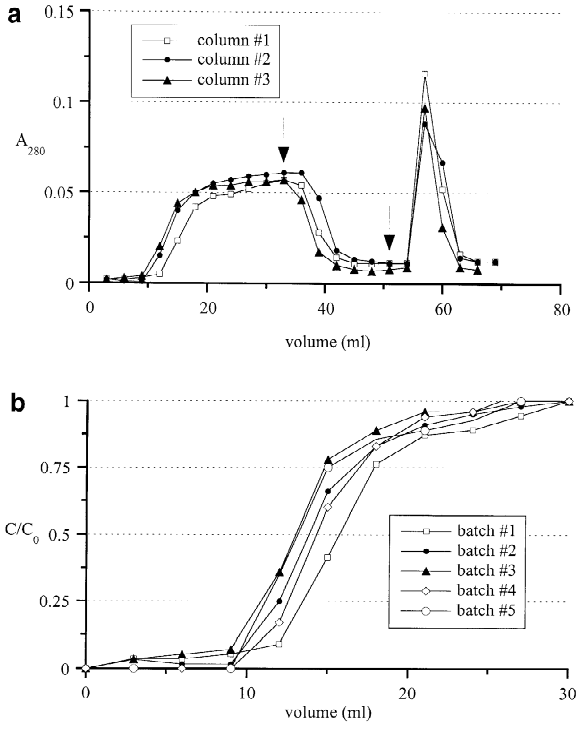

Fig. 4. Column-to-column variation within a batch (a) and batch-to-batch variation (b) for supermacroporous continuous columns. (a) Profile of BSA binding and elution for three columns taken randomly from the same batch. (b) Breakthrough curves of BSA for five columns taken randomly from different batches. Experimental conditions: 0.1 mg/ml BSA in 10 m*M* Tris–HCl buffer, pH 7.0 was applied to each column at a flow-rate 1 ml/min. First arrow indicates the start of washing with 10 m*M* Tris–HCl buffer, pH 7.0 and the second arrow indicates the start of elution with 1.5 *M* NaCl in 10 m*M* Tris–HCl buffer, pH 7.0.

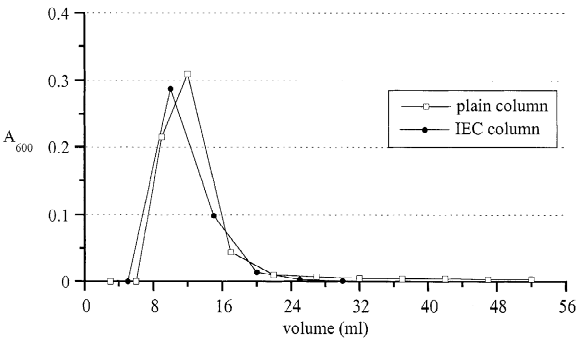

Fig. 5. Elution profile of *E. coli* cells from a plain supermacroporous continuous AAm/MBAAm-column (open squares) and supermacroporous continuous anion-exchange column under non-binding conditions (filled circles). Experimental conditions: 2 ml of *E. coli* cell suspension ($A_{600}=1.0$) in 20 m*M* HEPES, pH 7.0 containing 0.1 *M* NaCl (AAm/MBAAm-column) or 1 *M* NaCl was applied to an anion-exchange column at flow-rate 1.0 ml/min followed by washing with the respective buffer.

umns is technically straightforward and highly reproducible. As a test for the reproducibility in production of IEC-cryogel columns, from the six batches of 14 column in each batch, only a slight variation of BSA binding and elution profiles was observed for three columns taken randomly from the same batch (Fig. 4a). Batch-to-batch variation (Fig. 4b) also demonstrated a good reproducibility of produced anion-exchange adsorbents.

As mentioned earlier, the production and characterization of continuous supermacroporous columns bearing iminodiacetic acid ligands is described in detail elsewhere [10].

3.3. Chromatography of cells

The most attractive feature of polymeric cryogels from the bioseparation viewpoint is the supermacroporous structure formed due to the crystals of frozen solvent. Supermacropores in cryogels are of the 10–100 μm size and are also interconnected, not closed as in foam-like polymers e.g. foam rubber [14]. This pore morphology appears in cryogels due to the fact that crystals of the freezing solvent grow until they meet the faucets of other crystals [20].

E. coli cells with a size of 1×3 μm [21] were expected to pass rather easily through the pores of 10–100 μm size in continuous poly(AAm)-based supermacroporous matrix. In fact, no *E. coli* cells were retained on a plain AAm/Bis-AAm continuous supermacroporous column containing no ligands for specific interactions with cells. Also, on supermacroporous anion-exchange column in the presence of 1 *M* NaCl, sufficient to suppress electrostatic interactions, more than 95% of the cells were detected in the outflow (Fig. 5).

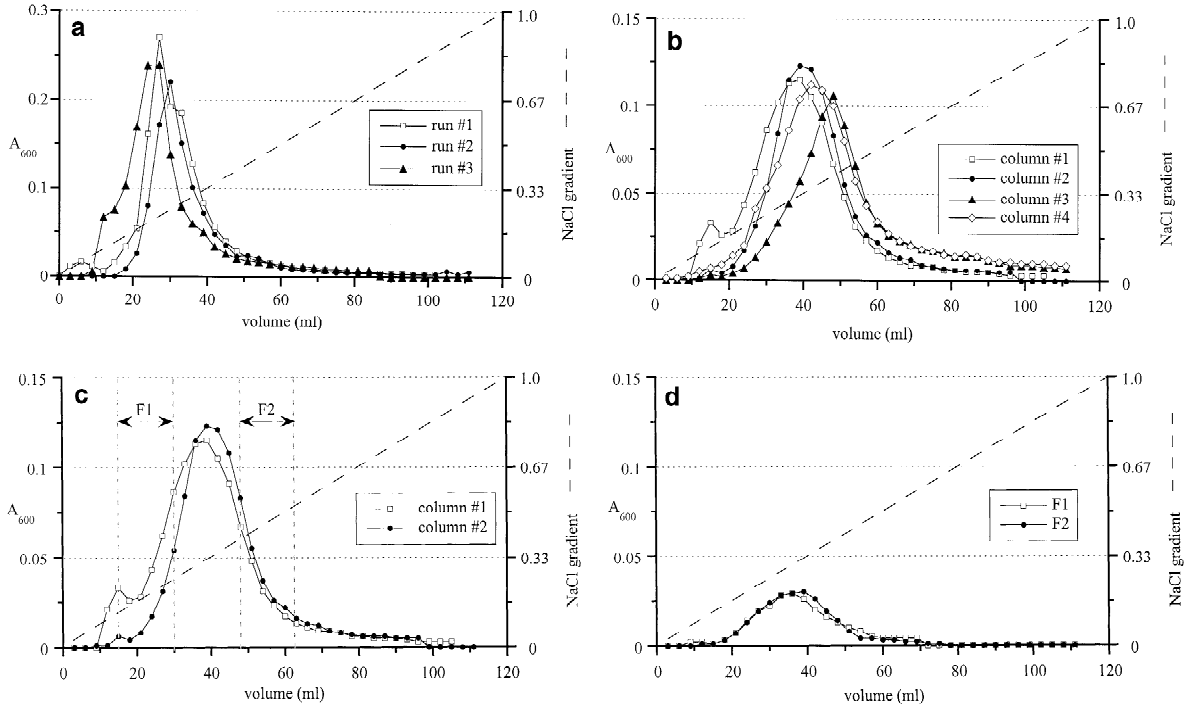

Fig. 6. Elution profile of *E. coli* cells from anion-exchange AAm/DMAEMA/BisAAm supermacroporous column. Experimental conditions: 3 ml of *E. coli* cell suspension with A_{600} of 1.2 was applied on a 5 ml supermacroporous anion-exchange column (length 45 mm, I.D. 12.4 mm) at a flow-rate of 1 ml/min and eluted with a linear 0.05–1.0 *M* NaCl gradient (120 ml) in 20 m*M* HEPES buffer pH 7.0. (a) Three samples of cell suspension applied on the same column. (b) Samples of cell suspension applied on different columns produced according to the same procedure (see Methods). (c) Dashed vertical lines indicate the collected fractions F1 and F2 from column no. 1 and no. 2 in b). Fractions F1 from the two runs and fractions F2 from the two runs were combined for re-chromatography. (d) Re-chromatography of fractions F1—open squares and F2—closed circles.

3.4. IEC of cells

The surface of *E. coli* cells is negatively charged and the cells are expected to bind to a positively charged carrier. Fig. 6a–d presents elution profiles of *E. coli* cells, which were applied on supermacroporous anion-exchange columns. The cells bound efficiently to the column at low ionic strength of 0.05 *M* NaCl and were eluted with 70–80% recovery at NaCl concentrations of 0.35–0.40 *M*. Run-to-run (Fig. 6a) and column-to-column variation (Fig. 6b)

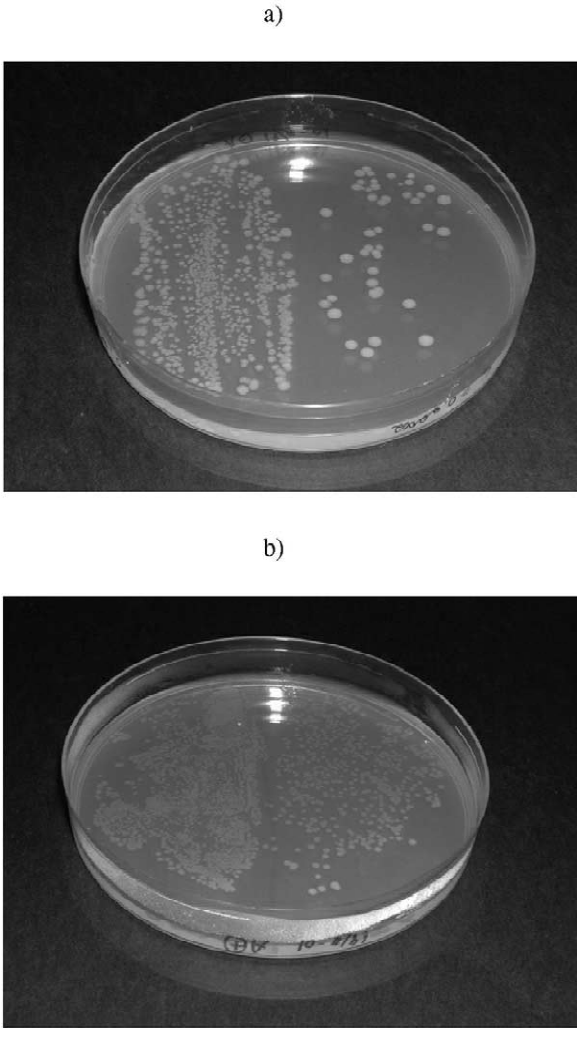

Fig. 7. Growth of *E. coli* cells 17 h at 37 °C on ampicillin-containing agar plates after (a) binding to a supermacroporous anion-exchange column and elution with NaCl gradient (as described in Fig. 6a). (b) binding to supermacroporous IMAC-column and elution with 20 m*M* EDTA pH 8.0 as described in Fig. 10a). For both (a) and (b): left side = eluted cells diluted 100-fold; right side = eluted cells diluted 1000-fold.

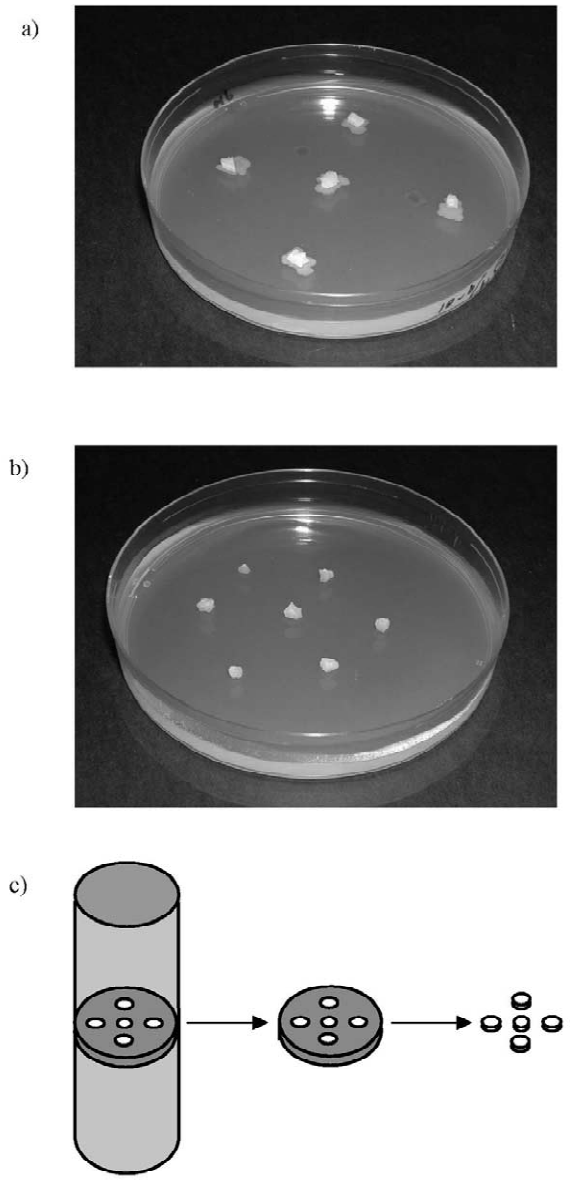

Fig. 8. (a) Growth from pieces of supermacroporous anion-exchange matrix on ampicillin-containing agar plates after binding ampicillin-resistant *E. coli* cells. (b) Pieces of a newly synthesized column on an agar plate without ampicillin. Both plates were incubated for 17 h at 37 °C. (c) Schematic explanation of how pieces of the supermacroporous matrix were taken.

demonstrated a reasonably good reproducibility of cell binding and elution. The collection (Fig. 6c) and re-chromatography (Fig. 6d) of early and lately eluted fractions indicated that all the cells in the sample behaved similarly from the chromatographic view point and the broadening of the peak was due only to the column properties. The cells eluted from the column retained their viability and grew on ampicillin-containing agar plates (Fig. 7a).

When cells are bound to the column, an important question is whether the binding takes place in the whole volume of the column or the cells are accumulated only at some particular zones, e.g. only at the top. One column was sacrificed after binding of ampicillin-resistant *E. coli* cells. The matrix was taken out from the column, the central disc-shaped zone was cut out and a few small pieces were taken from the central part of this disk and placed on top of an agar plate containing ampicillin. After incubation overnight at 37 °C, pronounced and approximately similar growth was observed around all pieces of the matrix exposed to cells (Fig. 8a). This indicates cell

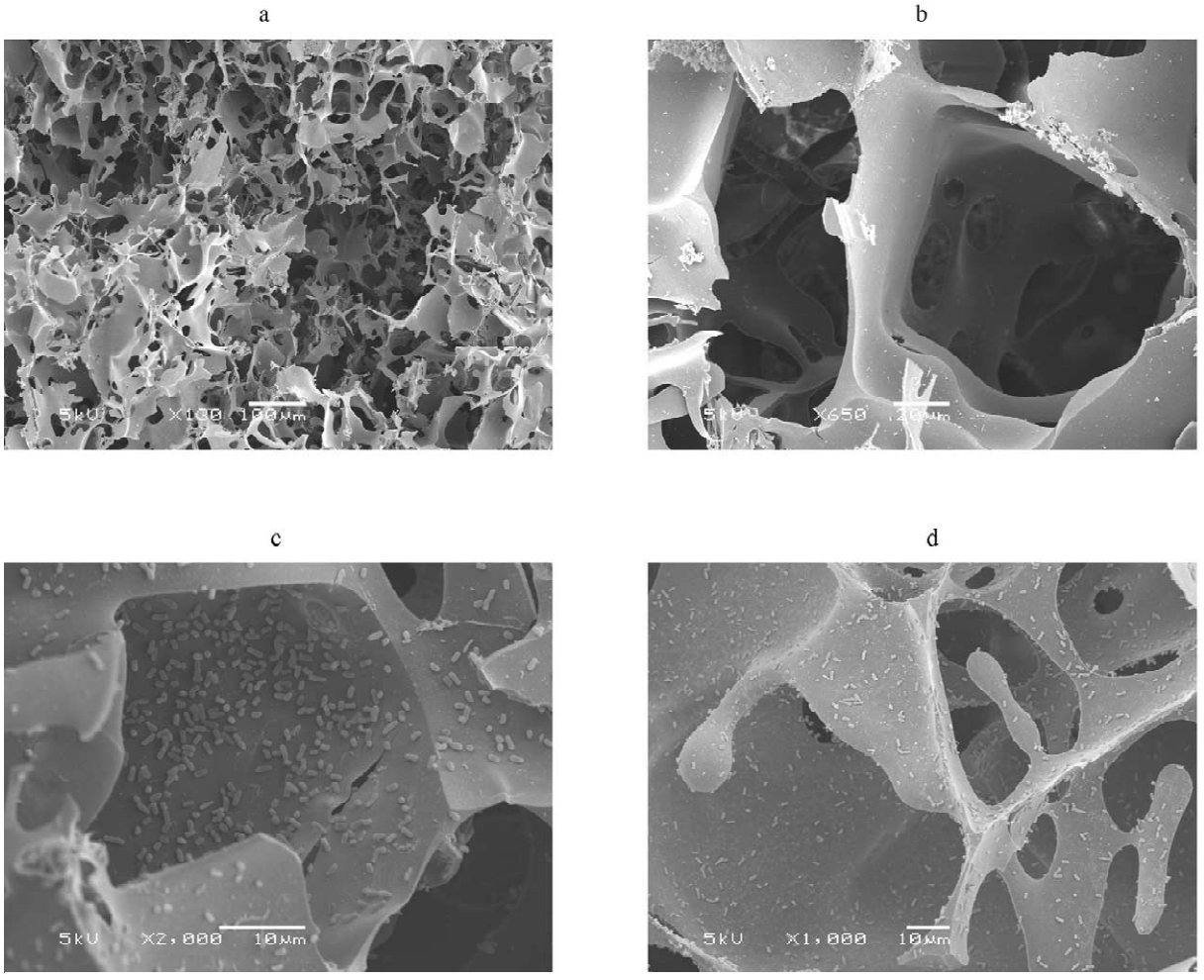

Fig. 9. Scanning electron microscopy photographs of supermacroporous anion-exchange matrix with bound *E. coli* cells at different magnification indicated by the bars at the bottom of the photographs. The samples were fixed in 2.5% glutaraldehyde in 0.15 *M* sodium cacodylate buffer pH 7.2 overnight, postfixed in 1% osmium tetroxide for 1 h, dehydrated in ethanol and critical point dried (see Methods). Dried samples were coated with gold/palladium (40/60) and examined using a JEOL JSM-5600LV scanning electron microscope. Cracks and crumbled material are artifacts of cutting and drying.

binding inside the whole volume of the supermacroporous anion-exchange cryogel column. As a control, pieces of an unused column were placed on top of an agar plate without ampicillin and no growth occurred under the same conditions (Fig. 8b).

Cell binding to the matrix was visualized using scanning electron microscopy (SEM) (Fig. 9a–d). The produced matrix is indeed supermacroporous consisting of large pores separated by rather thin polymer walls (Fig. 9a and b). Pore size of the matrix is much larger than the size of the cells, allowing them to pass easily. Bound cells are attached to the plain parts of the pore walls indicating specific interaction of cells rather than mechanical entrapment in ''dead flow'' zones.

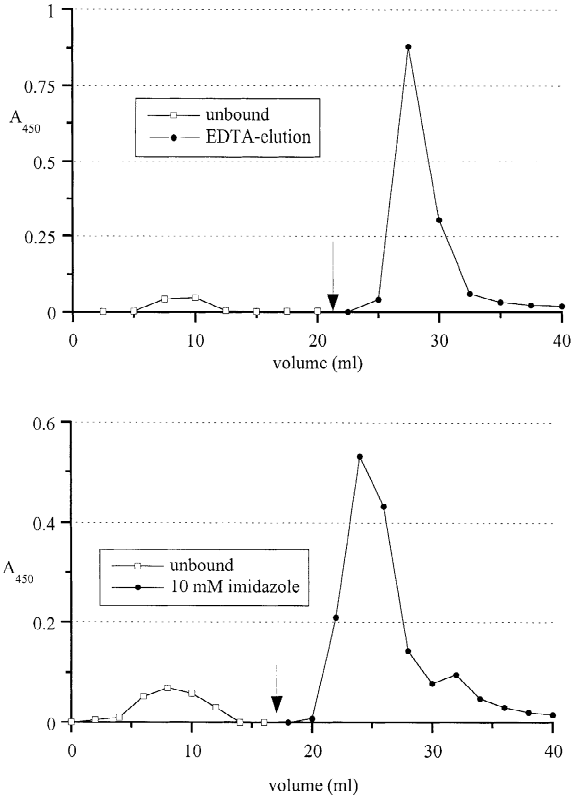

Fig. 10. Elution profiles of *E. coli* cells from supermacroporous IMAC column. (a) Elution with 20 m*M* EDTA, 50 m*M* NaCl, pH 8.0. Open squares represent unbound fractions, closed circles represent EDTA-eluted fractions and the arrow indicates start of elution. (b) Elution with 10 m*M* imidazole, 20 m*M* HEPES, 200 m*M* NaCl pH 7.0. Open squares represent unbound fractions, closed circles represent imidazole-eluted fractions and the arrow indicates start of elution. Experimental conditions: 4 ml of prefiltered *E. coli* cell suspension with A_{450} of ca. 1.0 was applied on a 5 ml supermacroporous IMAC column (length 45 mm, I.D. 12.4 mm) at a flow-rate of 1 ml/min.

3.5. IMAC of cells

E. coli cells have numerous proteins on the cell surface exposed into solution. One could expect that some of these proteins have inherent histidine residues accessible for the interaction with immobilized metal ions in the way similar to that of IMAC of proteins [22]. Indeed, *E. coli* cells were retained on a continuous supermacroporous column bearing iminodiacetic acid-ligands charged with Cu^{2+}-ions. No *E. coli* cells were eluted from the IMAC column with salt concentrations as high as 1.6 *M*, indicating specific character of cell binding to the matrix. Bound cells were eluted with nearly 80% recovery using stepwise increase in EDTA or imidazole concentration (Fig. 10a and b). Binding and elution procedure was mild and did not impair the viability of *E. coli* cells (Fig. 7b).

4. Conclusions

The results presented above indicate clearly that supermacroporous matrices produced by the cryotropic gelation technique provide an attractive tool for manipulating microbial cells in a chromatographic mode. With a proper choice of ligands capable of specific interactions with the surface of microbial cells, it is possible to bind cells quantitatively to the supermacroporous matrix and elute with recoveries as high as 70–80% without impairing viability of cells. It is reasonable to expect that this technique of cell chromatography can be expanded to the separation of cells differing in surface properties and hence in strength of interactions with immobilized ligands. Preliminary data support this assumption. Looking further, one could foresee the immunoaffinity chromatographic separation of different cell lines having specific antigens at their surfaces.

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