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Application of monoliths for plasmid DNA purification Development and transfer to production

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Abstract

The demand of high-purity plasmid DNA (pDNA) for gene-therapy and genetic vaccination is still increasing. For the large scale production of pharmaceutical grade plasmids generic and economic purification processes are needed. Most of the current processes for pDNA production use at least one chromatography step, which always constitutes as the key-step in the purification sequence. Monolithic chromatographic supports are an alternative to conventional supports due to their excellent mass transfer properties and their high binding capacity for pDNA. Anion-exchange chromatography is the most popular chromatography method for plasmid separation, since polynucleotides are negatively charged independent of the buffer conditions. For the implementation of a monolith-based anion exchange step into a pDNA purification process detailed screening experiments were performed. These studies included supports, ligand-types and ligand-densities and optimization of resolution and productivity. For this purpose model plasmids with a size of 4.3 and 6.9 kilo base pairs (kbp) were used. It could be shown, that up-scaling to the production scale using 800 ml CIM Convective Interaction Media radial flow monoliths is possible under low pressure conditions. CIM DEAE was successfully implemented as intermediate step of the cGMP pDNA manufacturing process. Starting from 200 l fermentation aliquots pilot scale purification runs were performed in order to prove scale-up and to predict further up-scaling to 8 l tube monolithic columns. The analytical results obtained from these runs confirmed suitability for pharmaceutical applications.

Keywords: DNA; Plasmid; Monoliths; Anion exchange chromatography; Gene therapy

1. Introduction

Although some technical and regulatory hurdles for DNA vaccines are reported to be still an issue [1] an increasing number of clinical trials for gene-therapy and genetic vaccination based on plasmid DNA (pDNA) reach the later clinical phases. The required amount of high-purity pDNA to feed these studies and finally the market have been underestimated in the past, since for clinical applications a trend from traditional vector systems, such as viruses, to safer but less efficient methods, such as naked pDNA and formulation as cationic complexes, can be observed [2–8]. Therefore industrial scale processes for the production of plasmids have to be suited for manufacturing grams or even several kilograms of purified pDNA per batch while meeting the appropriate quality standards requested by the national health agencies. Hence, the chromatographic supports used in such a process play a major role [9]. As productivity becomes a limiting factor chromatographic supports with a high dynamic binding capacity for pDNA are required.

pDNA applied as a DNA vaccine has to meet some typical quality specifications. In this context the product-quality is defined by the purity and the homogeneity as percentage of the supercoiled form compared to the total pDNA [10]. Supercoiled, also named covalently closed circular (ccc), pDNA [11] is the desired topological form since it induces the most

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efficient transfection and expression rate in eukaryotic cells. According to international regulations a content of ccc form higher than 90% is required [12–14]. Other undesired topological plasmid forms such as the open circular (oc) and the linear form, as well as dimers [15–17], reduce the homogeneity and should be removed by the separation process. Beside these, a variety of other host (*E. coli*)-related impurities such as genomic DNA (gDNA), RNA, proteins and endotoxins [23–25] have to be considered [8,9,18–22].

At laboratory scale, isolation of pDNA from crude cell lysates is well established [26,27]. For scientific purposes, simple commercial small-scale kits of different suppliers are available. They are designed for purifying small quantities of pDNA in the range of μg to mg, yielding a final preparation of minor quality [27]. This is sufficient for the majority of laboratory applications, of molecular cloning, but not for therapeutic purposes. Such pDNA purification processes consist of the following steps: cell lysis using lysozyme, RNA removal by RNase, extraction and precipitation with organic solvents and ultracentrifugation in density gradients. Due to their initial design, they are very time consuming and not scaleable. Other problematic issues are the use of flammable liquids, materials that are not certified for application in humans, enzymes from avian or bovine origin and toxic reagents such as phenol, CsCl or CsBr. To meet the appropriate guidelines of the regulatory authorities [2,28–30] such reagents have to be avoided in manufacturing of pharmaceuticals under current good manufacturing practice (cGMP) conditions.

An industrial manufacturing process for pDNA typically comprises fermentation, cell lysis, clarification, purification, polishing and final formulation and filling [2,21,30–32]. Liquid chromatography is considered as the downstream operation with highest resolution and is essential for pDNA production suited for therapeutic applications.

The requirements of a chromatographic support for pDNA separation are different from those for recombinant proteins, because these two classes of macromolecules differ significantly in their physico-chemical properties [33]. Plasmids are always negatively charged, are much larger in size and their shape resembles a long fiber. A typical plasmid is composed of 3–20 kilo base pairs (kbp), which corresponds to a relative molecular mass of 2×10^6–13×10^6 with a radius of gyration of 100 nm and higher [34]. The shape of the molecule was made responsible for the sensitivity against mechanical stress [9,35–38].

The isolation and purification of large polynucleotides, such as pDNA, is hampered by the low performance of commercially available chromatographic supports, which are mainly based on highly porous particles. Most of the chromatographic supports were tailor-made for the high adsorption capacity of proteins with a particle pore diameter of typically 30–400 nm, since proteins have diameters typically lower than 5 nm [34]. In columns packed with such supports, large molecules such as pDNA with a size of 100 nm to over 300 nm in diameter adsorb only at the beads outer surface [2,21,39–42]. Consequently capacities are on the order of tenths of milligrams plasmid per milliliter of chromatographic support compared to 200 mg/ml reported for proteins [2]. Thus, pDNA purification columns need to be large. Relatively low flow rates together with low capacity result in low productivities. Since most of the supports are reused a total pDNA recovery of about 100% after each cycle is mandatory to avoid carry-over from one purification batch to the next batch. Harsh cleaning conditions with up to 1 M NaOH are also preferred.

Traditional liquid chromatography is a rather slow, diffusion-controlled process. It often causes significant product loss due to oxidative degradation and enzymatic attack [43–45]. On the other hand, the efficient isolation of labile, valuable biomolecules requires a fast, reliable and affordable separation process under mild conditions.

For the purification of pDNA several chromatographic methods based on particulate supports have been reported [2,9,30,46]. Beside conventional techniques such as anion exchange [29], hydrophobic interaction [47] and size exclusion chromatography (SEC) [48] other methods were tested with more or less success. As examples triple-helix affinity [49], thiophilic interaction [50], reversed phase silica [51] or polymeric [52] and hydroxyapatite chromatography [53] have to be mentioned.

Alternatives to porous particles are the use of membrane- and monolith-technology, which reflect technological advances in fixed-bed liquid chromatography [54–56] based on favorable hydrodynamic properties compared to conventional supports. Membranes are very thin beds and can be considered as monolithic columns with an extreme aspect ratio. They provide a reduced pressure drop along the chromatographic unit, allowing increased flow rates and consequently higher productivity [54–56]. The problems with membranes are uniform flow distribution, a relatively large dead volume and scalability. To increase capacity membranes have been stacked into a column, which introduces additional void spaces.

A typical monolith is a continuous bed consisting of a single piece of a highly porous solid material [57,58]. Similar to membranes the most important feature of this support is that all the mobile phase is forced to flow through the large pores of the monolith [33]. As a consequence, mass transport is enhanced by convection, dramatically reducing the long diffusion time required by conventional particle-based chromatographic supports. Therefore, the chromatographic separation process on monoliths is practically not diffusion-limited [40,61–64]. The “large” channels (pores) of about 700–1000 nm of these monoliths allow binding of large molecules such as pDNA [43,65,66]. The high porosity of more than 50% leads to a low pressure drop [33].

Three types of monolithic separation-supports [59,60] are currently commercially available: Silica gel based monolithic beds [67] (Merck: “Chromolith”), polyacrylamide based monolithic beds [68] (Bio-Rad: “Uno”) and rigid organic gel based monolithic beds [69]. Polymethacrylate based short monolithic columns as stationary phases for

biochromatography [43] have been developed in the early 1990s. They are currently distributed under the trade name "Convective Interaction Media (CIM)" as disk monolithic columns and tube monolithic columns [70] (BIA Separations). It has been previously shown, that this material can be used for pDNA purification [40,43,66,71,72].

At laboratory scale a plasmid purification process using CIM columns was developed and implemented in a pilot scale. All critical elements of existing pDNA purification processes such as enzymes, detergents and organic solvents could be avoided. As a result a modern generic pDNA purification process which fulfills all regulatory requirements, delivering pDNA of high quality could be developed [31,73].

2. Experimental

2.1. Materials

The plasmid pRZ–hMCP1 [4.9 kbp; host: *E. coli* K12 JM108, ATCC No. 47107] and another model plasmid (6.9 kbp; host: *E. coli* K12 DH5-alpha, Invitrogen) were produced in the laboratory according to the procedure described in Section 2.2.1.

Purified pDNA for the determination of the dynamic binding capacity was also produced in the laboratory according to a modified laboratory-scale protocol [27] using a conventional chromatographic support. The purity of the pDNA solution was estimated to be around 75% at a content of 85% supercoiled pDNA of the total pDNA (homogeneity).

For the comparison studies DEAE Sepharose FF and Source 30Q were purchased from Amersham Biosciences (Uppsala, Sweden), Q Ceramic HyperD 20 from BioSepra (Cergy-Saint-Christophe, France), Fractogel EMD DEAE (S) from Merck (Darmstadt, Germany), Toyopearl DEAE 650 M from Tosoh (Stuttgart, Germany) and CIM DEAE disks from BIA Separations (Ljubljana, Slovenia). Dynamic binding capacity was determined with 1–2 ml of the particulate supports packed into columns with an inner diameter of 5 mm (Amersham Bioscienses). In the case of the monolithic support 1 CIM disk (diameter: 12 mm, height: 3 mm) mounted into a polypropylene housing (BIA Separations) was used. Further experiments were carried out with 8 ml (polypropylene housing) and 80 ml (stainless steel housing) CIM tube monolithic columns, provided by BIA Separarations. For the final up-scaling 800 ml cGMP CIM tube monolithic columns from BIA Separations were used. For capturing of pDNA from the clarified lysate Toyopearl Butyl 650 M (Tosoh) was bought. For the separation of precipitate and solution either a centrifuge (5810R, Eppendorf, Germany) or Kleenpack HDC II filters with 4.5 μm pore size, supplied by Pall (New York, NY, USA) were used. Ammonium sulfate was purchased from J.T. Baker (Phillipsburg, NJ, USA) and sodium dodecyl sulfate (SDS) from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were purchased from Merck. Ultradiafiltration was carried out by the Millipore Labscale TFF system (Millipore, Billerica, MA, USA) with 100 or 500 ml reservoir, using 1–3 regenerated cellulose 30 K membrane-casettes with 50 cm^2 area (Millipore, Billerica, MA, USA). Preparative chromatography was conducted on an Äkta Explorer 100 system consisting of a compact separation unit and a personal computer with Unicorn control system Version 4.00 or on the fast protein liquid chromatography (FPLC) system (both Amersham Biosciences, Uppsala, Sweden) at 254 nm. For up-scaling purposes chromatography units, which allow higher flow rates were used. The K-Prime 400 II system with software CCP Version 5.00 (Millipore, Billerica, MA, USA) therefore provides up to 3 l/min.

Analytical HPLC equipment consisted of an Agilent 1100 system (Agilent Technologies, Palo Alto, CA, USA) and a DNA-NPR column (i.d.: 4.6 mm, length: 75 mm, particle size: 2.5 μm) purchased from Tosoh.

2.2. Methods

2.2.1. Bacterial cell culture

A fermenter of a total volume of 30 l (continuous stirred tank reactor) was filled with the appropriate media compounds and deionized water to a final working volume of 20 l. The in house formulation consisted of complex components, a C-source, a macro elements and a trace elements solution. The total preculture volume of 200 ml was transferred into the fermenter and cultivation conditions were set as follows: aeration rate: 20 l/min = 1 vvm, agitation rate: 400–700 rpm (depending on dissolved oxygen tension), temperature: 37 °C, pressure: 0.5 bar, pH 7.0 (controlled with 25%, m/v, NH_4OH and 25%, m/v, H_2SO_4). The cultivation was terminated 12 h after the inoculation of the fermenter. After cooling down to lower than 10 °C, the culture broth was harvested and then separated by an ice water-cooled tube centrifuge. The obtained cell paste was packaged and stored at −70 °C.

2.2.2. Cell disintegration

The *E. coli* cells were disrupted by a modified alkaline lysis method initially described by Birnboim and Doly [74] either by manually carrying out the subsequent steps of the method or for larger amounts (>50 g) alternatively by a proprietary automated system [75].

The frozen cells were thawn and resuspended by adding 1 l of resuspension buffer (0.05 M Tris–HCl, 0.01 M EDTA, pH 8) to respectively 100 g bacterial cell paste and stirring till a homogenous suspension was obtained (about 1 h at room temperature). In a next step the resuspended cells were contacted and homogenously mixed with the same volume of lysis solution (0.2 M NaOH, 1% SDS) for 1.5–3 min. Neutralization took place by addition of an equal volume of neutralization solution (3 M potassium acetate at pH 5.5 at 4 °C) to the lysed cell solution. After homogenously and gentle mixing for 1.5–3 min. the mixture of pDNA containing lysate and the precipitated impurities (flocks) was separated. This clarification step was conducted by centrifugation at 7200 × *g*

for 10 min. or by the "clarification device" of the automated system.

2.2.3. Prepurification

As capture step hydrophobic interaction chromatography (HIC) was used. The chromatographic support was packed into XK columns from Amersham Biosiences or Moduline columns from Millipore with a packed bed height of about 20 cm.

To achieve binding of pDNA on this support the clarified lysate had to be conditioned. This was done by adding 2 l of a 4 M ammonium sulfate stock solution per 1 l of clarified lysate (resulting in 2.6 M ammonium sulfate in the sample) and contacting for at least 15 min. During the addition and the contacting the solution was slowly stirred. To obtain a clarified sample, which can be applied to the column the conditioned lysate was filtered. Loading of the column was performed at 150 cm/h while for elution the linear velocity was reduced to 75 cm/h. Elution was achieved by applying a linear gradient from 2.8 to 0.0 M ammonium sulfate in a buffer containing 0.1 M Tris–base and 0.01 M EDTA at pH 7. Supercoiled (ccc) pDNA containing fractions were pooled and further processed. The resulting pool was either directly used as sample for the anion-exchange chromatography (AIEC) step or after buffer exchange by ultradiafiltration to AIEC equilibration buffer (0.1 M Tris–base, 0.01 M EDTA, 0.3 M NaCl at pH 7). Alternatively the pool of the HIC-step was diluted with water to a conductivity lower than 40 mS/cm at room temperature.

2.2.4. Preparative anion-exchange chromatography

2.2.4.1. Dynamic binding capacity—screening of different supports. Several chromatographic supports for anion exchange chromatography were tested to determine their dynamic binding capacity for pDNA. This was carried out by recording breakthrough curves and calculating the bound amount of pDNA per ml support at 10% breakthrough. Each material was tested at different linear velocities.

The supports were packed into columns with a volume of 1–2 ml. For the CIM monoliths, disks (volume = 0.34 ml) mounted in a housing were used. Each chromatographic support was washed/regenerated with 0.5 M NaOH and subsequently equilibrated with loading buffer. The loading buffer contained 0.1 M Tris–base, 0.01 M EDTA and 0.3 M NaCl at pH 7. As load a stock-solution (1 mg/ml) of the purified 6.9 kbp plasmid was diluted with the loading buffer to about 55 μg/ml. This pDNA solution was loaded onto the particular chromatographic support till breakthrough (UV absorbance) stayed constant. The loaded amount of pDNA at 10% of the breakthrough UV absorbance indicated the dynamic binding capacity at the particular linear velocity for the tested support. Elution of the bound pDNA was achieved by increasing the NaCl concentration in the mobile phase to 1.5 M in a stepwise manner. Afterwards the supports were regenerated before the next run. Furthermore, the tested supports were compared regarding the back pressure at the end of the loading procedure and their productivity for pDNA. This economic parameter was calculated by comparing the amount of pDNA, which can be processed in a certain time range with a certain volume of chromatographic support according to the following equation [76]:

$$P = \frac{q}{Vt} = \frac{cyp}{t} \quad (1)$$

where P is the productivity (g/l h), q is the amount of purified product (g), V is the volume of the packed bed (l) and t is the time (h). Furthermore, c is the capacity of the support (g/l), y the yield of the chromatography step and p the purity of the product (both dimensionless; range: 0.0–1.0).

Yield in this case stands for the relative recovery of pDNA in the fractions, which fulfill the criterions to be pooled and further processed.

To simplify matters for the existing experiment and for comparison reasons yield and purity are assumed as 1 (100%), while the process time is assumed to be directly correlated to the applied linear velocity, which defines the throughput related to the same column diameter. Therefore, Eq. (1) simplifies to

$$P_0 = c_0 u_0 \quad (2)$$

where P_0 is a dimensionless productivity and c_0 and u_0 are the values for capacity and velocity as dimensionless relation parameters. A productivity factor P_f was used for comparison of the different chromatographic supports, by dividing the respective P_0 value with the highest P_0 value obtained within the series of experiments. This proceeding does not take into account the differences in loading time to reach the maximum capacity of each support at the specified velocity.

The experiments were repeated with CIM DEAE disks of different ligand density at one fixed moderate linear velocity (160 cm/h = 3 ml/min). Conversions factors of the tested disks (not commercially available) varied between 5.3 and 57.4%, corresponding to a ligand density of 220 μmol/g of dry monolith to 2000 μmol/g dry monolith. As sample the 4.9 kbp plasmid at a concentration of 100 μg/ml (initial sample diluted 1:10 with loading buffer) was used. The HPLC data were used to calculate pDNA recovery, which might be decreased using the disks with highest ligand density due to sticking on the column.

2.2.4.2. Ligand screening. Three different CIM AIEC ligands (standard conversion) were evaluated regarding their separation properties (resolution). As possible candidates two weak anion exchangers, CIM DEAE (diethylaminoethyl) and CIM EDA (ethylendiamine) were compared with the strong CIM QA (quarternary ammonium). As criterion the separation of the ccc pDNA from impurities and undesired pDNA forms was examined under the same conditions. For these experiments a HIC-prepurified pDNA solution, containing still a large amount of impurities, was diluted 1:10 with water to reduce the conductivity to a value comparable to the AIEC equilibration buffer (0.1 M Tris–base, 0.01 M EDTA,

0.3 M NaCl, pH 7). CIM disks with a volume of 0.34 ml were equilibrated for 30 column volumes (CVs) with AIEC equilibration buffer. The linear velocity was adjusted to 320 cm/h (= 6 ml/min = 17.6 CVs/min). Seventy millilitre of the diluted pDNA solution (c = 14 μg/ml) were loaded onto each disk. Afterwards, the disks were washed with 50 CVs of equilibration buffer. In a next step elution was carried out by a linear gradient of same slope for all disks. During the gradient the NaCl concentration in the buffer was increased from initially 0.3 M NaCl to finally 1.5 M NaCl. At the end regeneration by 0.5 M NaOH and 2 M NaCl was performed. For the evaluation of the specific support/ligand the UV absorption profile during elution was used as well as the analytical HPLC results (yield, recovery, homogeneity, estimated purity). The resolution R_s [77], which is defined as the distance between the centers of two eluting peaks as measured by retention time or volume ($v_2 - v_1$) divided by the average width of the respective peaks [$(w_2 + w_1)/2$]:

$$R_s = \frac{v_2 - v_1}{(w_2 + w_1)/2} \quad (3)$$

was calculated by the Unicorn software of the chromatographic workstation. Baseline separation and thus 100% peak purity is achieved at an R_s value greater than 1.5 (R_s = 1 means 98% purity, assuming 98% peak recovery). Since at 254 nm UV absorption reached the upper detection limit the similar curve at 280 nm was used to evaluate accurate values.

2.2.4.3. Optimization. The influence of different flow rates on the performance of the CIM DEAE disks was investigated. Therefore, one disk was mounted into the housing and varying flow rates for all chromatography steps were applied. After equilibration with the equilibration buffer (buffer B) 35 ml of prepurified pDNA solution (HIC Pool with a large amount of impurities diluted 1:10 with water) was loaded at flow rates between 15 and 30 ml/min (equivalent to 800–1600 cm/h). The pDNA concentration was about 11 μg/ml. Elution was achieved at the same flow rate by a linear gradient from 0% to 60% of the elution buffer (buffer B) (A: 0.3 M NaCl, 0.1 M Tris–base, 0.01 M EDTA, pH 7/B: 1.5 M NaCl, 0.1 M Tris–base, 0.01 M EDTA, pH 7) in 50 CVs. For the evaluation the separation of the impurity peak from the ccc pDNA peak (resolution), as well as the retention volume and the peak areas were taken into account. Furthermore, the peak width was determined in order to estimate and compare the expected pDNA concentration in the eluate.

Further optimization work was focused on the improvement of the separation by gradient optimization. As a first approach a detailed investigation of the linear gradient took place. Therefore, the influence of different gradient length was tested. In this series samples adequate to the previously described one were used as feed solution. The linear velocity was fixed at 320 cm/h for all steps and experiments. Elution was achieved by applying the same linear gradient as described for the varying linear velocities (0–60% buffer B). The gradient length varied between 40 and 160 cv.

The same setup was used to establish the step gradient, suitable to separate impurities and undesired pDNA forms from supercoiled pDNA. Based on the results of the gradient optimization work several different combinations of salt concentrations (and therefore conductivity) were tested as specific step elution operations. The performance of the first elution step was tested at 50, 55 and 60 mS/cm, while for the second step buffers with a conductivity of 60 and 70 mS/cm were applied. To remove stronger bound material a third step at 100% B (125 mS/cm) was used and finally a regeneration step carried out with a combination of 2 M NaCl and 0.5 M NaOH. The linear velocity was constant at 320 cm/h. The peak areas of the resulting peaks were compared to the peak areas of the optimized linear gradient. The peaks were collected and the fractions analyzed by HPLC in order to determine if they contain ccc pDNA.

After optimization of the previous steps the optimized step gradient was applied for elution of pDNA from an 8 ml CIM DEAE tube monolithic column. The applied flow rate was 20 ml/min for loading and 10 ml/min for elution. The HIC pool was loaded directly without any adjustment (no dilution or ultradiafiltration) at high conductivity (ammonium sulfate concentration). The main fractions of the peak were analyzed by HPLC.

2.2.4.4. Scale up. For the scale up 8, 80 and finally 800 ml CIM DEAE prepacked radial flow tube monolithic columns were used (Fig. 1). The chromatographic parameters were calculated according to the following equations. Since these

Fig. 1. Shape of CIM tube monolithic columns for radial chromatography in different dimensions (80 and 800 ml are commercially available; the 8000 ml monolith is a prototype).

larger units are prepared as radial flow columns a changing linear velocity along the chromatographic bed has to be taken into account. The mobile phase velocity at the inner and at the outer surface of the tube monolith is calculated by

$$u = \frac{F}{A} \tag{4}$$

and

$$A = D\pi L \tag{5}$$

with u as the mobile phase velocity (cm/min), F as the volumetric flow rate (ml/min), A as the surface area (cm^2) at the inner and at the outer side of the radial flow column (A_o and A_i), L as the length/height (cm) of the tube and D as the respective diameter (cm) (measured from the center) at the inner and at the outer side of the monolithic layer (D_o and D_i). The order of increase of the linear velocity from the outside to the inner channel of the monolith can be described by the ratio of the linear velocities at the respective surface (u_i/u_o).

For the average linear velocity it follows [78]:

$$u_{av} = \frac{F}{\pi L}\frac{\ln(D_o/D_i)}{(D_o - D_i)} \tag{6}$$

For the transfer between different sizes of monolithic columns gradient time for constant resolution can be calculated according to a simple equation [79,80]:

$$t_{g,large} = t_{g,small}\left(\frac{V_{large}}{V_{small}}\right)\left(\frac{F_{small}}{F_{large}}\right)\left(\frac{L_{small}}{L_{large}}\right) \tag{7}$$

where t_g is the gradient time (min), V the respective total column volume (ml) and F is the flow rate (ml/min). In this equation L has to be considered as the length of the monolithic bed (cm), where the separation takes place, which is described by the thickness of the monolithic layer in the case of radial flow columns. The transfer of gradient chromatographic methods of Convective Interaction Media monolithic columns is described by Zmak et al. [79].

To investigate possible deviations in the results at different scales of CIM DEAE supports experiments with 8, 80 and 800 ml columns were carried out. These tests were part of a scale comparison study of the whole process including also the other process steps at the respective scale. Therefore the methods of prepurification before the AIEC step were similar but consequently the AIEC load not exactly the same. The loaded pDNA amount per liter of support was in the same range for all experiments. The runs on the 80 ml and on the 800 ml column were performed in triple repetition to verify reproducibility. The applied flow rates were increased according to the column volume, in order to maintain the process time constant (same cv/min) for the step elution. The obtained chromatograms were compared for similarity of the elution profile, while the collected pDNA fractions (main peak) were analyzed for homogeneity and purity. Finally the HPLC data were used to calculate recovery and yield.

Productivity of the support at the different scales was calculated by comparing the amount of pDNA, which can be processed per hour and liter of chromatographic support according to Eq. (1).

2.2.5. Analysis/analytical methods

For the quantification and qualification of pDNA an in house developed anion exchange HPLC protocol was used as standard method. Since it enables to distinguish between the different topological pDNA isoforms the homogeneity can be determined beside the pDNA concentration. Furthermore it delivers also a rough estimation of the impurity content by comparing the total peak area of all pDNA isoforms, which bind on the column, with the peak area of non-binding impurities.

The applied flow rate for the DNA-NPR column is 1 ml/min for all steps. As equilibration buffer 20 mM Tris–HCL at pH 9.0 is applied. Prior to injection most of the samples were diluted 1:2 with water to reduce the salt concentration to a value, which allows binding on the analytical column. Depending on the pDNA concentration, between 5 and 30 μl of the sample were injected. Elution was achieved by applying a linear gradient of 50–75% elution buffer (20 mM Tris–HCl, 1 M NaCl, pH 9.0) within 5 min. Detection was carried out at a wavelength of 260 nm (reference: 360/100).

For the analysis of impurities standard methods were used. Genomic DNA was determined by Southern blotting and RNA by agarose gel electrophoresis. For the detection of residual protein the bicinchoninic acid (BCA) assay was used. Endotoxins were analyzed by a LAL-gel clotting assay according to USP.

3. Results and discussion

In order to get a first impression about the suitability of different support types for an economic pDNA production process their dynamic binding capacity was evaluated. The characteristics of the tested supports are summarized in Table 1. All materials listed here do meet the regulatory guidelines for production of biopharmaceuticals. They can be sanitized by NaOH. The particle diameter ranged from 20 to 90 μm except for the monoliths with an apparent particle diameter of 1.5 μm. The pore size was in the range of 2–200 nm for the beads, while monoliths have an internal channel diameter of 1500 nm. Another objective was to determine the range of applicable linear velocities for each support. These data were finally used to calculate the productivity of the supports. The results are summarized in Fig. 2, which shows the correlation between the mobile phase velocity and the dynamic binding capacity for each tested support in comparison. Concerning these parameters the supports can be classified into two groups. A group with a generally low capacity of about 0.5–0.8 mg/ml at 100 cm/h could be observed (DEAE Sepharose FF, Toyopearl DEAE 650 M and Source 30Q). Another group (CIM DEAE, Fractogel EMD DEAE (S) and Q Ceramic HyperD 20) showed a dynamic binding capacity for pDNA in the range of about 3–9 mg/ml, which is

Table 1
Characteristics of the anion-exchange supports evaluated with respect to their pDNA binding capacity

Support	Functional group	Matrix	Particle size (μm)	Pore size (nm)	Ligand density (μeq./ml)
DEAE Sepharose FF	DEAE	Agarose	90	190	180–250
Source 30Q	Q	Polystyrene divinylbenzene	30	2–100	NA
Q Ceramic HyperD 20	Q	Hydrogel/ceramic	20	NA[a]	150–400
Fractogel EMD DEAE (S)	DEAE	Methacrylate	40–90	80	NA
Toyopearl DEAE 650 M	DEAE	Methacrylate	40–90	100	80–120
CIM DEAE	DEAE	Methacrylate (Monolith)	1.5[b]	700–950[c]	2500

[a] Gigapores of rigid ceramic bead, filled with hydrogel.
[b] Apparent particle diameter calculated by Zöchling et al. [40].
[c] Channel radius.

one order of magnitude higher compared to the first group of supports. While the two particulate supports are described by decreasing capacity with increasing linear velocity the CIM material maintained a high capacity even at increased mobile phase velocities (1000 cm/h). This observation confirms the results obtained at lower linear velocities (up to 300 cm/h) reported by Zöchling et al. [40]. The behavior can be explained by the structure of the supports. While the materials of the first group due to their pore structure behave like non-porous beads [40–42] the number of binding sites accessible for pDNA in the Fractogel material is increased, because of the tentacular construction. The high capacity of Ceramic HyperD 20 was tried to be explained by partitioning of pDNA to the hydrogel filling the pores of the ceramic skeleton [29]. The dependence of dynamic binding capacity with velocity of Fractogel and the Ceramic HyperD support is explained by diffusional limitations, whereas mass transport in monolithic columns is mainly driven by convection and thus not diffusion limited. This can be explained by the structure, in case of the CIM supports, the high porosity and the 1.5 μm channels through the solid material, which do not restrict the transport. Since the dynamic binding capacity of the monolithic support was highest also the pDNA-concentration of the elution peak

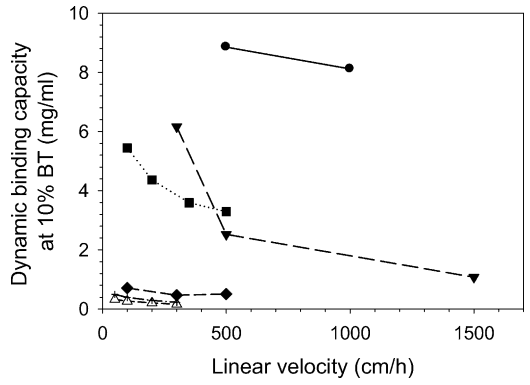

Fig. 2. Comparison of different supports (1–2 ml, 0.34 ml disk) regarding dynamic binding capacity (10% breakthrough) for pDNA. Feed stock: purified pDNA solution. Source 30Q (♦), Toyopearl DEAE 650 M (+), DEAE Sepahrose FF (△) represent a class providing low capacity. Q Ceramic HyperD 20 (▼) and Fractogel EMD DEAE (S) (■) showed a higher dynamic binding capacity for pDNA at the lower linear velocities. CIM DEAE (●) was the only support which provided a high capacity (>8 mg/ml) at high flow rates (500–1000 cm/h).

was higher than in the other cases. The pDNA is eluted in a lower volume. This is an important advantage, if size exclusion chromatography follows after AIEC. Loading volume of SEC is limited and considering the overall process-economy a low elution volume from AIEC has a big impact.

Since degradation of pDNA is also time dependent, especially due to enzymatic digestion in fractions of lower purity, the primary and intermediate steps of a pDNA production process should be conducted as fast as possible. Therefore, another criterion for the choice of the support was process time. In addition pressure drop is another limit. The pressure drop at the end of loading at a medium and a high linear velocity, corresponding dynamic binding capacity and the productivity for each support are summarized in Table 2. The comparison of the productivities, calculated according to Eq. (2), clearly show the advantage of the monolithic material from the economic point of view. Since the flow rate has only a minor impact on capacity it can be chosen independently. During loading on the CIM DEAE disk an increasing back pressure was observed. At a linear velocity of 500 cm/h 0.4 MPa were measured at the start of loading and 1.2 MPa at the end. The columns filled with beads did not show this effect, except Fractogel, which showed a slight increase. The pressure drop increase during loading of pDNA on monoliths was also described by Zöchling et al. [40], who explained it by a gradual filling of the pores with plasmid DNA. The free cross sectional area available for the liquid flow is reduced, resulting in a reduced permeability and hence an increased pressure drop. This behavior especially at higher amounts of loaded pDNA has to be taken into account for the choice of the chromatographic system used at the large scale. When high linear velocities should be run, medium pressure systems are recommended. The monolith itself is stable up to 3 MPa pressure drop and the housing of larger units are manufactured of stainless steel. A higher pressure drop is therefore not an issue.

CIM DEAE was selected for further examinations as intermediate step in our pDNA production process due to the productivity and good recovery, yield and resolution observed in preliminary experiments with HIC-prepurified sample (data not shown).

To further optimize the properties of the CIM DEAE monolith, disks with different ligand density were tested for their dynamic binding capacity for pDNA at 160 cm/h. A

Table 2
Comparison of supports regarding pressure drop, capacity and productivity at a medium and a high linear velocity

Support	Linear velocity (cm/h)	Pressure drop (MPa)	Dynamic binding capacity (mg/ml)	Productivity factor P_f (dimensionless)
DEAE Sepharose FF	100	0.21	0.263	0.003
	300	0.28	0.157	0.006
Source 30Q	100	0.11	0.707	0.009
	500	0.32	0.505	0.031
Q Ceramic HyperD 20	300	0.30	6.162	0.228
	500	0.37	2.521	0.155
Fractogel EMD DEAE (S)	100	0.61	5.443	0.067
	500	0.71	3.287	0.203
Toyopearl DEAE 650 M	100	0.22	0.390	0.005
	300	0.36	0.225	0.008
CIM DEAE	500	1.20	8.856	0.546
	1000	1.30	8.116	1

The higher pressure drop on the monolith also results from the high amount of pDNA loaded and should be considered for the large scale.

linear relationship between ligand density and capacity was observed within a range (Fig. 3). This indicates that even at the highest ligand density (47.4% conversion) additional ligands are not affected by steric hindrance and probably binding capacity therefore can be further increased by increasing the conversion. At the lowest ligand density (conversion: 5.3%) the dynamic binding capacity at 10% breakthrough was determined with 1.6 mg pDNA/ml support, while at 47.4% 7.1 mg/ml could be achieved. Similar results were reported by Bencina et al. [72] for genomic DNA 50 kbp. The breakthrough curves shown in Fig. 4 represent examples for a low, a medium and a high ligand density. In all cases the profile is steep, which is advantageous and results from the fast convective transport mechanism. All the tested prototypes showed a high recovery of about 95%, taking regeneration not into account. Thus we assumed a CIM disk or column could be completely regenerated.

While DEAE groups showed good results, before final selection of the matrix, also other available anion-exchange ligands were tested in order to find the optimal one regarding resolution and capacity. We tested three types, DEAE, QA and EDA, respectively with feed solutions containing a high amount of impurities. When comparing the elution profiles (Fig. 5) it can be seen that the CIM modified with DEAE and with QA ligands showed a resolution of two peaks, while the elution profile of CIM EDA showed only one maximum. The first peak mainly contained impurities and pDNA of low homogeneity, mostly linearized and/or oc pDNA were present. The second peak contained desired pDNA, the ccc topology in a high concentration. Although the concentration of impurities was high in the feed solution and the column loaded to a high extent in this experiment, resolution of 1.31 was obtained with DEAE CIM disks, while the monolith with QA groups showed a low resolution of $R_s = 0.56$ (Table 3). CIM EDA showed lowest resolution and lower capacity, since breakthrough was observed (Fig. 5C). All collected pools showed constant or improved homogeneity.

For further optimization CIM DEAE columns were selected. In order to find out the influence of velocity on the elution profile, test runs were performed at velocities from 800 to 1600 cm/h. The superimposed profiles are shown in

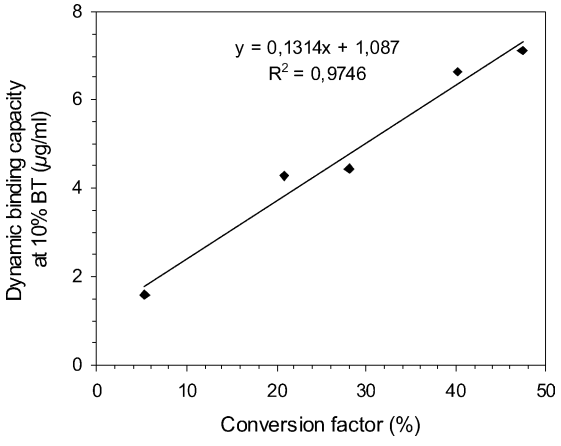

Fig. 3. Dynamic binding capacity at 10% breakthrough in dependency of the ligand density of specially prepared CIM DEAE disks. Feed solution: purified pDNA solution. A linear relationship between ligand density and dynamic binding capacity for pDNA can be recognized.

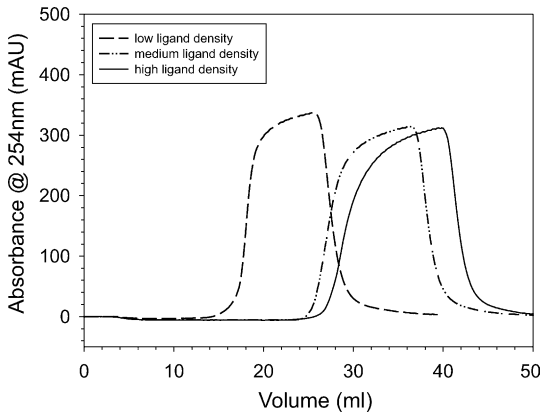

Fig. 4. Selected breakthrough curves of CIM DEAE disks with low, medium and high ligand density.

Table 3
DEAE, EDA, two weak anion-exchange ligands and QA, a strong one, compared regarding their resolution, the purity parameters (homogeneity, estimated purity), an economic parameter (pool yield) and a cleanability parameter (recovery)

Ligand	Resolution, R_s	Pool homogeneity (% ccc)	Estimated purity (%)	Pool yield (%)	Recovery (%)
DEAE (diethylaminoethyl)	1.31	78	92	100	100
EDA (ethylendiamin)	None	88	33	60[a]	92
QA (quarternary ammonium)	0.56	85	79	75	97

[a] Thirty one percent of the loaded pDNA amount was found in the flow through fraction, due to overloading and breakthrough.

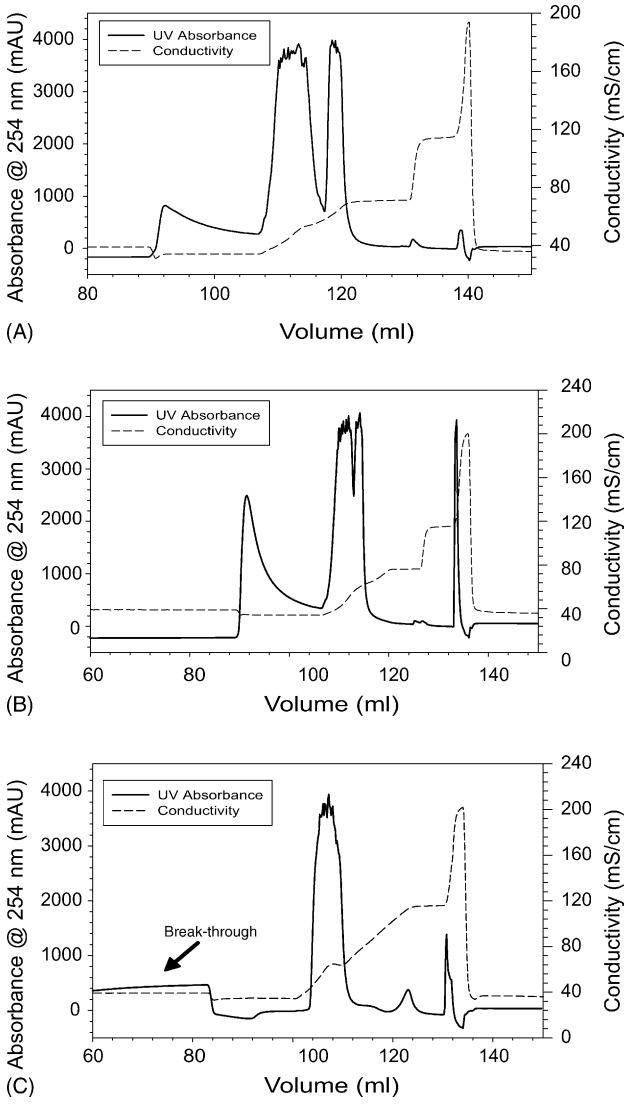

Fig. 5. Comparison of different anion exchange ligands on CIM disks in respect to resolution. (A) DEAE, (B) QA, (C) EDA.

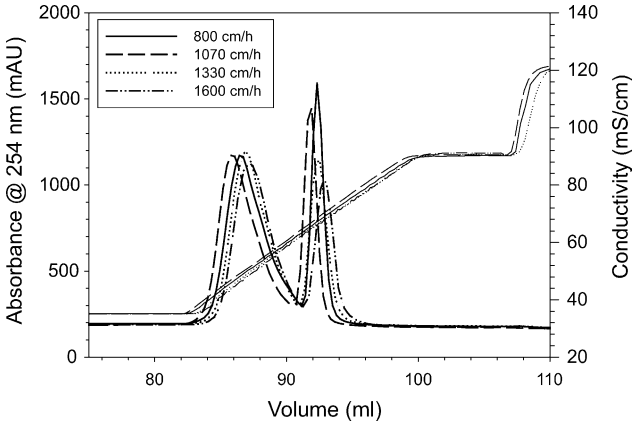

Fig. 6. Influence of linear velocities during elution on the resolution (CIM DEAE disk).

Fig. 6. The profiles do not change with velocity. The ratio of peak areas was constant; a value of 70:30 was observed in all cases (see Table 4). Peak position and peak width were not affected by the linear velocity. The maximal applicable mobile phase velocity was defined between 1300 and 1400 cm/h, which did not yet cause a peak broadening effect. It should be

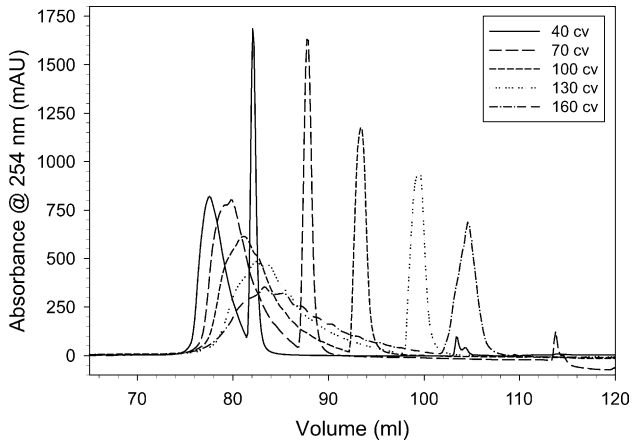

Fig. 7. Elution profiles at different gradient slopes (CIM DEAE disk).

Table 4
Influence of linear velocities on the ratio of peak areas and the total peak width

Linear velocity (cm/h)	Flow rate (ml/min)	Area% peak 1	Area% peak 2	Total peak width (ml)	Retention volume peak 2 (ml)
800	15	68.71	31.29	12.17	92.35
1100	20	68.56	31.44	14.28	91.89
1400	25	68.35	31.65	13.79	92.41
1600	30	68.68	31.32	15.35	92.96

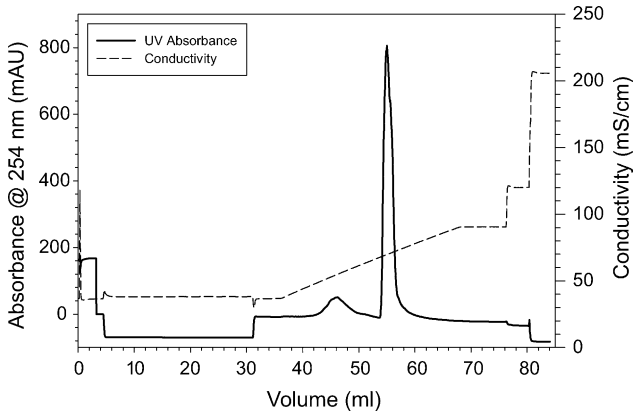

Fig. 8. Elution profile by optimized linear gradient elution (CIM DEAE disk).

noted, that this linear velocity corresponds to about 70 CVs and furthermore theoretical calculations for a 10 l column, suitable for loading of more than 50 g plasmid, resulted in a volumetric flow rate of 700 l/min. Such a volumetric flow rate can practically never been reached by currently available chromatography systems.

The next objective of our work was gradient optimization. The experiments were performed to find out, at which gradient length baseline separation of the impurity peak from the pDNA peak can be achieved. Fig. 7 shows the obtained elution profiles, indicating that baseline separation is possible

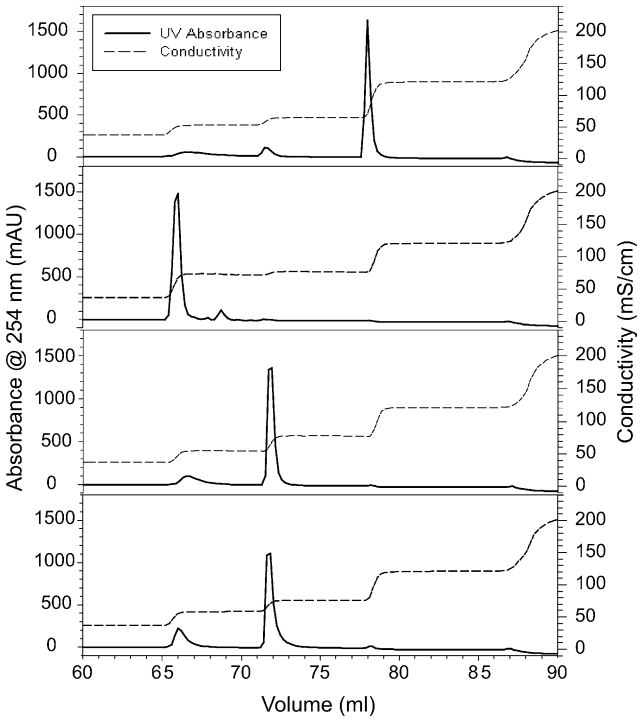

Fig. 9. Elution profiles at different step gradients (CIM DEAE disk). For the evaluation of the four different combinations, elution profiles and ratio of peak areas were compared with the result of the optimized linear gradient elution (Fig. 8).

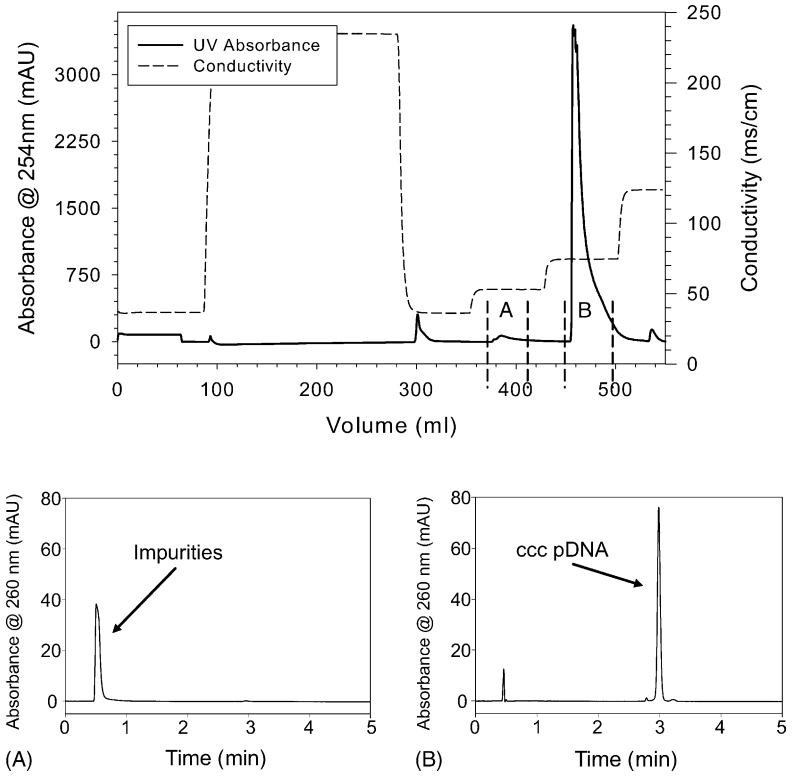

Fig. 10. Performance of the optimized AIEC step performed on an 8 ml CIM DEAE tube monolithic column using step gradient elution. The upper chromatogram shows the preparative elution profile, while the lower two show the results of the HPLC analysis of fractions (A) and (B).

at a gradient length of 130 cv or longer. According to theory peaks become broader with shallower gradient. The salt concentration at which a compound elutes from a conventional ion exchanger is dependent on the steepness of the gradient [81,82]. A significant change of salt concentration of peak maxima at different gradient slopes was not determined (peak maxima in the range of 67.86–68.29 mS/cm). Podgornik et al. [83] observed with CIM disks, that salt concentration of peak maxima is not significantly affected by the gradient slope. This was explained by the high ligand density of CIM disks. Based on these facts and the elution profile obtained with the further optimized linear gradient (Fig. 8), the salt concentration at the peak maximum was selected as basis for the step elution experiments.

The step gradient test series (Fig. 9) revealed, that a conductivity of the first step of 55 mS/cm or higher, already caused elution of a substantial amount of pDNA together with impurities in this step. For the second elution step 70 mS/cm is optimal for getting high yield of pDNA without stripping impurities with higher affinity from the column. For this model plasmid with 6.9 kbp the optimal elution steps were a step gradient with 50 mS/cm to elute impurities and some undesired pDNA forms, followed by a second step at 70 mS/cm for the elution of ccc pDNA. Using the same feedstock, the comparison with the linear gradient elution (Fig. 8) confirms that the ratio of peak areas is in the same range. Yields greater than 90% with constant homogeneity (data not shown) could be obtained. For plasmids strongly differing in size, adjustment of the specific elution steps will be necessary. Binding strength to an anion-exchanger is a function of charge, which is higher in larger plasmids.

Finally, the obtained conditions were transformed for the operation of 8 ml radial flow CIM tube monolithic column. Since it was found out during the optimization that the pool from the previous step (HIC) can be directly loaded onto the CIM AIEC column at high ammonium sulfate concentration without further handling, this procedure was established for all further experiments. Anyway, binding of pDNA on the CIM DEAE support at conductivities higher than 200 mS/cm was not observed when NaCl was additionally present in the feed solution. When the NaCl concentration in the washing buffer was higher than 0.3 M (about 35 mS/cm) early elution of pDNA at the beginning of the washing procedure was observed. Optimizing the HIC capture step (data not shown) the amount of impurities loaded onto the AIEC monolith column could be minimized, resulting in smaller impurity peaks in the CIM DEAE run. The result of a purification run conducted with an 8 ml CIM tube and the previously optimized step gradient is shown in Fig. 10 as elution profile for the 4.9 kbp plasmid as example; similar results were obtained with the 6.9 kbp plasmid. Furthermore Fig. 10 shows the corresponding HPLC analysis of the peak fractions. The first peak (A) contains only impurities and the large second peak (B) contains the highly purified pDNA. The amount of supercoiled pDNA (retention time of about 3 min) is much higher compared to the impurities (at about 0.5 min). Resulting homogeneities, after this step were reproducibly greater than 95%. Due to the high capacity of the monolithic column the plasmid concentration of the eluate was also very high. A

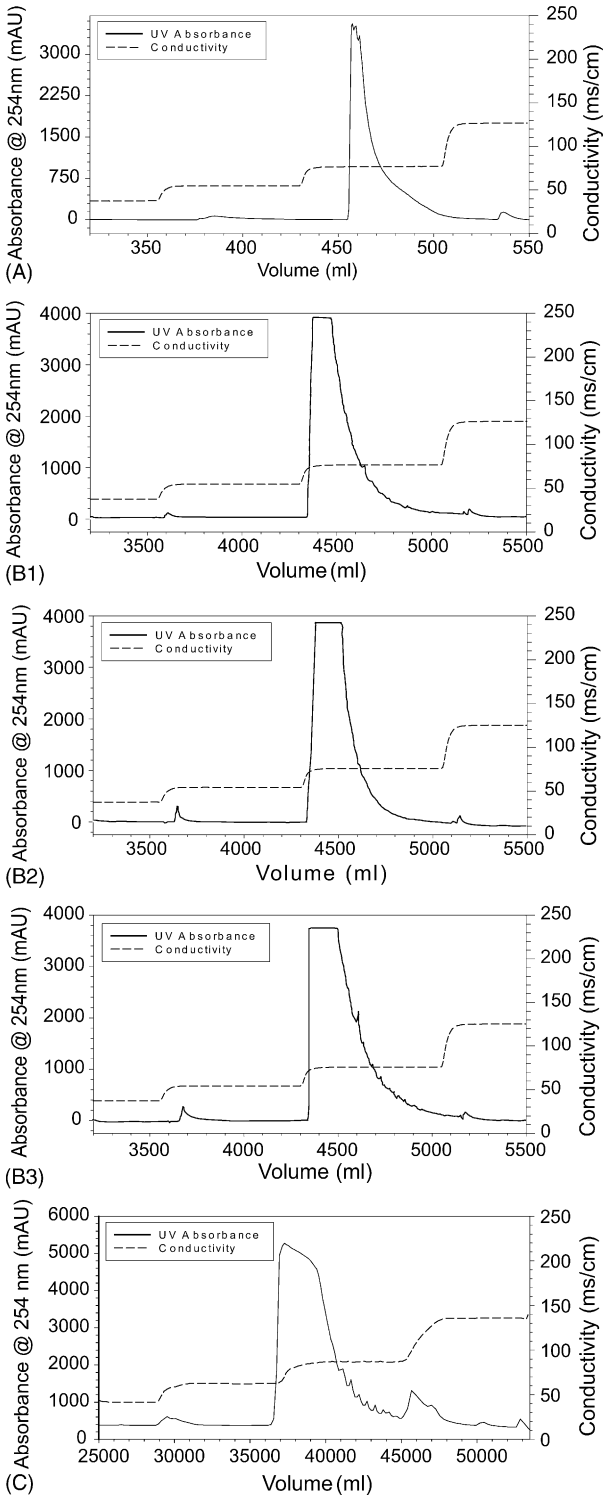

Fig. 11. Elution profiles at different scales in comparison showing scalability and reproducibility of CIM DEAE supports. (A) 8 ml tube monolithic column, (B1)–(B3) Three subsequent runs on the same 80 ml tube monolithic column, (C) 800 ml monolithic column.

Table 5
Dimensions of CIM Tube monolithic columns

Volume of stationary phase (ml)	Amount of pDNA processed (mg)	Outer diameter (cm)	Inner diameter (cm)	Layer thickness (cm)	Length (cm)
8	50	1.5	0.15	0.68	4.5
80	500	3.4	1.5	0.95	11
800	5000	11	7.2	1.90	15
8000	50000	30	25.6	2.20	41.5

The 8000 ml tube is under development and was not tested during this work.

Table 6
Linear velocity parameters of different sizes of radial flow tube monolithic columns used for development and up-scaling

Volume of stationary phase (ml)	Operating flow rate (ml/min)	Linear velocity at the outer surface (cm/h)	Linear velocity at the inner surface (cm/h)	Average linear velocity (cm/h)	Ratio of inner: outer linear velocity
8	20	56.6	566.0	144.6	10.00
80	200	102.2	231.6	149.6	2.27
800	2000	231.7	353.4	283.8	1.53
8000[a]	20.000/10.000	307.2/153.6	360.0/180.0	331.2/165.6	2.34/1.17

The 8000 ml tube is under development and was not tested during this work.
[a] The first value corresponds to the linear up-scaled flow rate, while the second value is valid for the decreased (50%) flow rate.

pool concentration between 0.5 and 1.0 mg pDNA/ml could be obtained.

The objective of our development work was the implementation of the AIEC step for larger scales. Up-scaling to 800 ml units via the intermediate size of the 80 ml radial flow column was performed [80,83]. The dimensions of the monolithic units are specified in Table 5 and the shape of radial columns is shown in Fig. 1. Since resolution and binding capacity of the monoliths is flow rate independent the scale-up approach of radial chromatography with varying linear velocities at the inner and at the outer surface is possible. Scale-up was performed by keeping process time constant and all process steps such as feed, wash, elution were scaled in respect to total column volume. From flow rates and column dimensions (Table 5) inner, outer and average linear velocities were calculated by Eqs. (4)–(6). The outer and average velocity increase, while inner velocity is between 230 and 570 cm/h (Table 6). Chromatograms (Fig. 11) and product and impurity analysis (Table 7) confirm that linear scale up of the flow rate with the column size at the same step gradient gives identical results. Especially the endotoxin levels were low in all obtained pools. In Fig. 11 the elution profiles of the runs performed at the different scales are shown. When scaled to column volume all runs are comparable to each other. Due to the higher amount of pDNA loaded per ml support, the main peak is broadened compared to the small scale run displayed in Fig. 10. Since the pDNA elutes in a very high concentration (>2.5 mg/ml) the upper detection limit is reached in the second elution step. For all runs impurities in the collected pool were in the same range. The slight difference in yield is explained by the fractions subjected to the pool and the quality of the initial feed solution. From a pragmatic point of view it was not possible to use the same feedstock for all scales. The results do not only show scalability and robustness but also reproducibility. The comparison of the shown analytical data of three consecutive runs performed with 80 ml

Table 7
Comparison of analytical results of the CIM AIEC intermediate step at different scales using 8, 80 (three repetitions) and 800 ml CIM DEAE tube monolithic columns in the respective scale of the process

Parameter	Column size				
	8 ml	80 ml—1	80 ml—2	80 ml—3	800 ml
Flow rate (ml/min)	20	200	200	200	2000
Loaded pDNA amount (g/l)	4.7	4.7	3.4	4.8	5.2
	AIEC pool analysis				
Concentration (g/l)	0.92	0.81	0.59	1.06	0.96
Homogeneity (% ccc)	93.4	97.3	97.2	97.6	92.9
Purity ($A_{260}/A_{280\ nm}$)	NA	1.90	1.92	1.91	1.90
Approximate purity by HPLC (%)	97.7	93.1	92.6	92.1	96.8
gDNA (ng/μg pDNA)	<25	<25	<25	<25	<25
RNA (ng/μg pDNA)	<20	<40	<40	<40	<20
Protein (ng/μg pDNA)	85	137	141	94	76
Endotoxin (EU/μg pDNA)	$<1.2 \times 10^{-4}$	$<1.8 \times 10^{-3}$	$<1.2 \times 10^{-2}$	$<2.1 \times 10^{-3}$	$<6.2 \times 10^{-4}$
Yield (%)	86.3	96.1	88.1	98.4	87.3
Recovery (%)	97.5	99.8	97.4	100	>92.1[a]

[a] Wash and regeneration fractions were not taken into account.

units shows only minor deviations between the runs. With the exception of protein content, in all experiments a pDNA solution meeting the individually required quality criterions for a pharmaceutical pDNA product was obtained already after this second chromatography step carried out by the CIM DEAE supports. Nevertheless, an additional step for further protein reduction and desalting for final formulation is still needed.

The productivity of the AIEC step of 8.7 g pDNA/l h was calculated according to Eq. (1) using the following parameters: capacity was 6.3 g/l, yield was 90%, purity was 92%, process time was 0.6 h; only time for loading, washing and elution was included. The corresponding value for a conventional support run at 150 cm/h with 1 g/l capacity and a packed bed height of 15 cm is about 0.6 g/l h, assuming same data for purity and yield as for the monolith. This result confirms an about 15 times higher productivity of the monolithic support. Even if a support with a same capacity like monoliths is used and the applicable mobile phase velocity is doubled (300 cm/h) productivity would not exceed 4 g/l h, which is still less than half of the CIM.

For further up-scaling to 8000 ml columns it is recommended to reduce the flow rate to a maximum of 10 l/min since a higher back pressure is expected due to increased thickness (bed depth) of the monolithic layer, which is needed to increase the volume.

4. Conclusions

The high capacity of the CIM supports for the chromatographic purification of pDNA at high flow rates was the intention to set up a down-stream process based on CIM monoliths. They exert a high productivity, due to excellent mass transfer properties and a large number of accessible binding sites for pDNA, which both result from the monolith specific structure. Furthermore the selected AIEC monolith exerts a good resolution, which is maintained also at increased linear velocities. CIM supports are sanitizeable, certified and thus suited for pharmaceutical production. For the pDNA purification these novel chromatographic supports represent an advantageous alternative to conventional supports due to these features. It could be shown, that up-scaling to the production scale using 800 ml radial flow monoliths was possible under low pressure conditions. CIM DEAE was successfully implemented as intermediate step of the cGMP pDNA manufacturing process, providing the same results as shown during the development. For larger amounts of purified pDNA (0.5–1 kg) further up-scaling can be envisioned.

Acknowledgments

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