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Application of short monolithic columns for fast purification of plasmid DNA

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Abstract

Anion-exchange chromatography is one of the most important methods in downstream processing of plasmid DNA, both as a process and as an analytical technique. Separation of plasmid DNA on traditional particle-based anion-exchange supports is usually slow. Moreover, such supports have a low capacity for plasmid DNA due to the steric exclusion effects. In this work, the separation of plasmid DNA using short monolithic columns, Convective Interaction Media, will be presented. It will be demonstrated that plasmid DNA can be purified from bacterial cells using alkaline lysis followed by chromatography on a very short weak anion-exchange chromatographic columns—disks—with good purity and quality within a short time. Furthermore, the separation of plasmid DNA from cell RNA can be carried out without the need of adding RNAse. Fast and efficient method for *in-process* control of the purified plasmid will be described as well.

Keywords: Purification; Monolithic columns; Plasmid DNA

1. Introduction

Recent developments in molecular biology have resulted in new technologies for the production of complex biomolecules. One of the most important and most cost-intensive steps is the isolation and purification of the target molecules. Plasmid DNA is a high molecular mass compound, the purification of which for pharmaceutical use is usually not an easy task to perform. Plasmids are circular duplex DNA molecules that are stable maintained as episomal genetic information within bacteria [1]. Replication of plasmid DNA does not depend on any plasmid-encoded protein and is not synchronous with replication of the bacterial host chromosome [2]. Plasmids vary widely in their copy number (from 1 to 1000 copies per cell) depending on the origin of replication, size of the plasmid and its associated insert. They may appear in different forms: circular with different degrees of coiling, partially cleaved or linear, and multimeric as concatamers or catenates [3]. Plasmid vectors are usually produced in *Escherichia coli* by fermentation. They are isolated mostly by an alkaline lysis procedure, designed to disrupt cell walls and denature proteins and genomic DNA followed by one or more purification steps [4]. With the increasing utilisation of plasmid DNA, there is a growing need for simple and robust purification processes that can be used in the isolation of plasmid DNA from transformed bacteria. To be able to use plasmid DNA as a therapeutic agent, it is essential that it is free of any other materials. Possible contamination components include substances used in the isolation process and material from host cells, mainly residual proteins, RNA, and genomic DNA [4].

Today, there are a lot of different approaches to purify plasmid DNA. Some of them are based on methods such as high salt precipitation of RNA in a DNA concentrate [5] while the others are based on chromatographic separations [6–11].

In general, high-performance liquid chromatography (HPLC) is the most important tool for the analytical and preparative separations of biomolecules. In the last 15 years several attempts have been made to develop adsorbents that would allow fast and efficient separation of large

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biomolecules such as plasmid DNA. The crucial factor for a successful and fast separation of such molecules, which are characterised by their large molecular radius and thus low molecular diffusion coefficient, is a reduced mass transport resistance within large open-end pores/channels as described by various authors like, e.g. Tennikova et al. [12,13] for high-performance membrane chromatography on porous monolithic disks or Lightfoot and co-workers [14] and Etzel and co-workers [15] on stacked-membrane columns based on polyethersulfone.

Monolithic supports represent a novel type of chromatographic stationary phases introduced in the past 15 years [16–20]. The main difference between monoliths and conventional porous particles is in their structure. The monoliths are a single piece of highly porous material characterised by a highly interconnected network of channels with a diameter in the range of 10–4000 nm or even larger [21]. Consequently, mass transport in monoliths is mainly based on convection, the basis for naming one particular type of these supports as Convective Interaction Media CIM® [22]. Since large channels can easily accommodate large plasmid DNA biomolecules, the CIM® monoliths have a very high binding capacity for plasmid DNA that can reach up to 10 mg/ml [21]. Furthermore, for the optimal separation performance of high molecular weight biomolecules, the chromatographic column needs to be short. This further enhances the speed of the separation process and reduces the backpressure, unspecific binding and product degradation, without sacrificing resolution [21].

In this paper, an application of CIM® monolithic columns for the purification of plasmid DNA is described. Furthermore, because of the fast and efficient separation of RNA from DNA, a potential application of CIM® DEAE for the *in-process* control of DNA is presented. Plasmid pcDNA 3.1 (5.4 kb) was chosen as a model for purification on monolithic columns throughout the experimental work.

2. Experimental

2.1. Chemicals

E. coli DH5α and agarose were obtained from Life Technologies Ltd. (Paisley, UK). Plasmid pcDNA 3.1 (5.4 kb) was from Invitrogen (Groningen, The Netherlands). Luria–Bertani (LB) medium was from Institute of Immunology Inc. (Zagreb, Croatia). Ampicilin was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Tris(hydroximethyl)methylamine, ethylene-diamine tetraacetic acid (EDTA), sodium dodecylsulfate (SDS), isopropanol, boric acid and ethydium bromide were purchased from Sigma (St. Louis, MO, USA). Ethanol and 3-[*N*-morpholino]propansulfonic acid (MOPS) were obtained from Amresco (Solon, OH, USA). QIAGEN plasmid mini kit was from QIAGEN GmbH (Hilden, Germany).

2.2. Instrumentation

All column chromatography experiments were performed using ÄKTA™ purifier (Amersham Biosciences, Uppsala, Sweden).

Electrophoresis of plasmid DNA was performed on the electrophoretic unit HE 33 (Hoefer, San Francisco, CA, USA).

Spectrophotometric determination of plasmid DNA concerning to purity and yield was performed by BioPhotometer (Eppendorf AG, Hamburg, Germany).

2.3. Columns

CIM® Convective Interaction Media® disk monolithic columns bearing weak (diethylaminoethyl (DEAE)) anion group were used throughout the experimental work (BIA Separations, Ljubljana, Slovenia). CIM® monolithic column consists of a disk-shaped poly(glycidyl methacrylate-co-ethylene dimethacrylate) highly porous polymer matrix that is seated in a non-porous self sealing fitting ring. The bed volume of one disk is 0.34 ml, and the porosity is 62%. The disk-shaped matrix is inserted in commercially available PEEK or polyacetal housing (BIA Separations) and connected to an HPLC system. Regeneration of CIM® DEAE disk monolithic column was performed after each run by washing it with 10 column volumes of 2 M NaCl followed by 10 column volumes of 0.5 M NaOH at a flow rate of 4 ml/min.

2.4. Methods

2.4.1. Transformation of *E. coli* and overnight fermentation

Transformation-competent *E. coli* DH5α cells were transformed with the pcDNA3.1 (5.4 kb) using a method previously described [23]. Overnight cultures of colonies were grown in LB medium supplemented with ampicilin (100 μg/ml), in 500 ml shake flasks at 37 °C.

2.4.2. Alkaline lysis of *E. coli*

Alkaline lysis of *E. coli* was based on the method of Birnboim [24] with modifications as indicated. Overnight cultures (2 ml) were centrifuged at 4000 × *g* for 3 min at 4 °C in a microfuge. The bacterial pellet was resuspended in 100 μl of ice-cold 50 mM glucose/25 mM Tris–HCl/10 mM EDTA, pH 8.0. Suspension of cells was incubated for 5 min at room temperature and 2 min on ice. A volume of 200 μl of a freshly prepared solution containing 0.2 M NaOH and 1% SDS were added to the suspension of cells and mixed. After 5 min of incubation on ice, 150 μl of 3 M potassium acetate (pH 5.7) was added in the suspension and incubated 5 min on ice. After incubation, the mixture was centrifuged at 14000 × *g* for 20 min at 4 °C. The supernatant was collected and applied on monolithic units.

Flow through and eluted fractions were precipitated by 1 volume of isopropanol for 15 min on ice and centrifuged at 14000 × *g* for 10 min at 4 °C. The pellet was washed by 70% ethanol and centrifuged again at 4000 × *g* for 10 min. The pellet was air-dried for 5–10 min and dissolved in 50 μl of 10 mM Tris–HCl (pH 8.5).

Plasmid DNA prepared by this way was tested by restriction digestion analysis, by transformation experiments and by agarose gel electrophoresis. Determination of yield and plasmid DNA purity was performed by measuring absorbance at 260 (A_{260}) and 280 (A_{280}) and expressed as $A_{260/A280}$ ratio in a low-salt buffer. As a positive control, the supercoiled plasmid DNA purified by a commercial QIAGEN plasmid mini kit was used.

2.4.3. Purification of plasmid DNA using monolithic disks

A short monolithic column (CIM® disk) bearing weak (DEAE) anion-exchange groups was used for the purification of plasmid DNA. The column was first equilibrated with 25 mM MOPS, 0.5 M NaCl, pH 7.0 binding buffer, containing 15% isopropanol (v/v). The low concentration of alcohol in the buffer eliminates non-specific hydrophobic interaction [25]. After that, 0.4 ml of plasmid DNA, already pre-treated by alkaline lysis, was applied on the disk at the flow rate of 4 ml/min. Non-bound proteins and nucleic acids were washed out with 10 ml of 25 mM MOPS buffer, 0.5 M NaCl, pH 7.0 containing 15% isopropanol (v/v). Bound nucleic acids were eluted with 25 mM MOPS, 1.5 M NaCl, pH 7.0 buffer containing 15% isopropanol (v/v). Elution was carried out using linear (0–50% buffer B in 1.7 min) and stepwise gradients (at 10, 20 and 50% buffer B, each step lasted for 0.68 min). Non-bound material, fractions collected during washing step as well as fractions eluted by increasing of salt concentration were collected and further analysed.

2.4.4. In-process control of plasmid DNA using disk monolithic column

After the purification of plasmid DNA on a disk monolithic column, the isolated DNA was desalted by precipitation with isopropanol (see protocol in Section 2.4.2). After that, 30 μl of precipitated plasmid DNA was applied again on the equilibrated DEAE disk monolithic column. The binding and elution buffers were the same as used in the first chromatography step. As a control, plasmid DNA isolated by QIAGEN purification kit was also applied on the same disk monolithic column. Non-bound macromolecules were washed out with the binding buffer at a flow rate of 4 ml/min. Bound macromolecules were eluted with a stepwise gradient at 10, 20 and 50% buffer B, where the duration of each step was 0.68 min.

2.4.5. Agarose gel electrophoresis

Agarose gel electrophoresis was carried out by using a horizontal 1.0% agarose slab gel in 89 mM Tris, 89 mM boric acid and 2 mM EDTA, pH 8.3. The electrophoretic separation was performed at a constant voltage at room temperature for 0.5 h. Plasmid DNA was stained with a solution of 1 μg/ml ethidium bromide included in the gel.

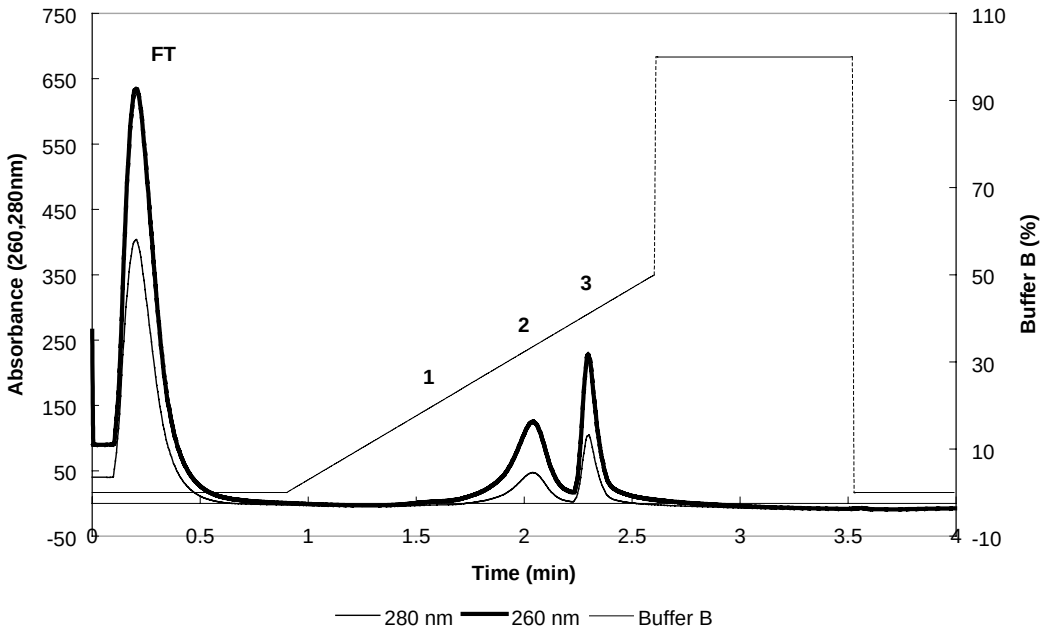

Fig. 1. Purification of a plasmid pcDNA 3.1 on CIM® DEAE disk monolithic column using linear gradient elution. Binding buffer: 25 mM MOPS, 0.5 M NaCl, pH 7.0, 15% isopropanol (v/v); elution buffer: 25 mM MOPS, 1.5 M NaCl, pH 7.0, 15% isopropanol (v/v); flow rate: 4 ml/min; gradient: 0–50% linear in 1.7 min. (—) Absorbance at 280 nm; (▬) absorbance at 260 nm; (· · ·) increased concentration of buffer B. FT: flow through; peaks 1–3 are the collected fractions.

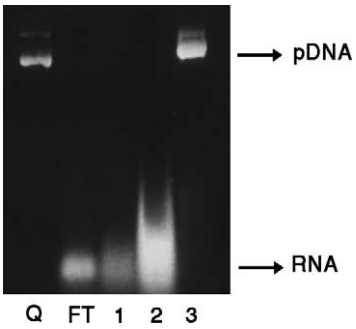

Fig. 2. Ethidium bromide-stained agarose gel of the fractions collected during chromatography of pcDNA 3.1 plasmid on CIM® DEAE disk monolithic column (Fig. 1, linear salt gradient). Q: plasmid DNA isolated by QIAGEN purification kit; FT: flow through collected during chromatography described in Fig. 1; (1–3): fractions collected according to chromatographic profile in Fig. 1.

3. Results and discussion

The most common methods for the initial isolation of plasmid DNA are modified versions of two approaches: one is based on a release of the plasmid by boiling [26] and the other one is based on an alkaline treatment and detergent-mediated solubilisation of the bacterial cell membranes [27]. Both of these methods result in the release of plasmid DNA from its cytosolic location. In both cases downstream purification involved either ultracentrifugation using a caesium chloride (CsCl) density gradient, selective precipitation of plasmid from contaminants and/or the use of diverse clean-up procedures based on DNA-binding matrices. Since the technique involving CsCl density gradient centrifugation is expensive, time consuming, and involves a substance that is toxic for the environment and the product, it is not favourable for laboratory and large-scale purification of plasmid DNA.

Anion-exchange chromatography has become fundamental in the downstream processing of plasmid. In this work, the use of a monolithic chromatographic media with a weak anion exchanger ligand, Convective Interaction Media CIM®, was tested. Purification of plasmid (pcDNA 3.1, 5.4 kb) on a strong anion (quaternary ammonium (QA)) disk monolithic column tested at first was not entirely successful since it was not possible to completely separate RNA from DNA, as observed by electrophoresis on agarose gel (data not shown). A possible reason could be a stronger binding of RNA on a QA disk monolithic column compared to a DEAE disk monolithic column.

Purification of pcDNA 3.1 on a DEAE disk monolithic column within 5 min using a linear gradient with a salt concentration from 0 to 50% buffer B in 1.7 min is shown in Fig. 1. Agarose gel electrophoresis of different fractions collected during this chromatographic run is presented in Fig. 2. A very clear and sharp band of plasmid DNA is found only in fraction 3 while a smear of RNA is obtained in flow through and in fractions 1 and 2 (Fig. 2, lanes FT and 1–3). As a control, pcDNA 3.1 plasmid DNA purified with a commercial QIAGEN plasmid kit was used (Fig. 2, lane Q). In the next step, the chromatography using stepwise gradient was carried out. According to the elution profile of plasmid DNA in a linear gradient, the best conditions for elution of bound

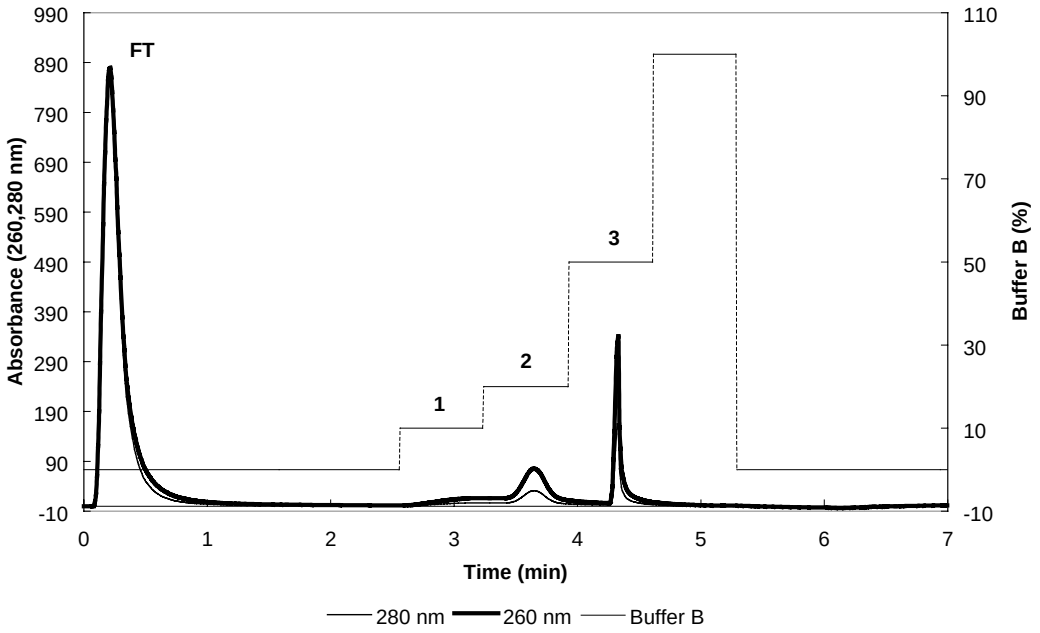

Fig. 3. Purification of a plasmid pcDNA 3.1 on CIM® DEAE disk monolithic column using stepwise gradient elution. Binding buffer: 25 mM MOPS, 0.5 M NaCl, pH 7.0, 15% isopropanol (v/v); elution buffer: 25 mM MOPS, 1.5 M NaCl, pH 7.0, 15% isopropanol (v/v); flow rate: 4 ml/min; stepwise gradient: 10, 20 and 50% steps (each step takes 0.68 min). (—) Absorbance at 280 nm; (▬) absorbance at 260 nm; (···) increased concentration of salt. FT: flow through; peaks 1–3 are the collected fractions.

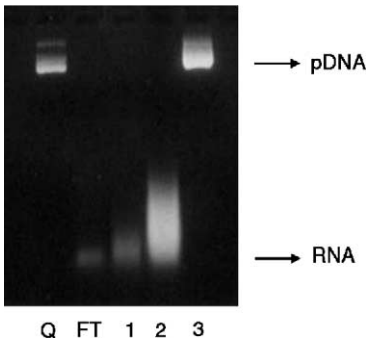

Fig. 4. Ethidium bromide-stained agarose gel of the fractions collected during chromatography of pcDNA 3.1 plasmid on CIM® DEAE disk monolithic column (Fig. 3, stepwise gradient). FT: flow through collected during chromatography described in Fig. 3; (1–3): fractions collected according to chromatographic profile in Fig. 3. Q: plasmid DNA isolated by QIAGEN purification kit.

nucleic acids by stepwise gradient were chosen as follows: the first peak was eluted with 10% buffer B, the second with 20% and the third with 50% buffer B. Although DNA could be eluted using a lower salt concentration, 50% buffer B was chosen to accelerate the elution. In Fig. 3, a chromatographic profile of such a stepwise gradient is presented. Non-bound nucleic acids were marked as FT fraction (flow through). During the run, fractions were collected and precipitated with isopropanol as described in Section 2.4.2. Although the separation time was 7 min, even faster separation is possible depending on the technical possibilities of the used HPLC system. In Fig. 4, the agarose gel electrophoresis of the corresponding precipitated fractions is presented. As shown in Fig. 4, flow through and fractions eluted with 10 and 20% buffer B (see chromatogram in Fig. 3, FT, fractions 1 and 2) show a smear of RNA. However, the fraction eluted with 50% buffer B (peak 3), contains pure pDNA. Compared to DNA purified by QIAGEN, the quality of plasmid DNA was satisfactory. This was further confirmed by fast rechromatography (within 4 min) of plasmid DNA (*in-process* control) on a CIM® DEAE disk monolithic column. In previous works, a fast *in-process* control during downstream processing of protein mixtures has already been presented [28–31]. In this work, a similar procedure has been applied to *in-process* control during downstream processing of plasmid DNA. After the purification step, the quality of the eluted plasmid DNA fraction was controlled by fast rechromatography on the same disk, as an *in-process* control. Moreover, plasmid DNA purified by the QIAGEN purification kit was used as a control and also injected on the CIM® DEAE disk. Fig. 5 shows the two superimposed chromatograms. Plasmid DNA purified by QIAGEN purification kit has a very sharp peak eluted with 50% buffer B (DNA) and a broader peak eluted with 20% buffer B. A similar chromatogram was obtained using pDNA purified on a monolithic column. In spite of the fact that the QIAGEN kit included RNAse in a standard protocol for plasmid DNA purification, traces of RNA were confirmed in the purified plasmid DNA. Agarose gel electrophoresis confirmed RNA in both fractions eluted with 20% buffer B (Fig. 5, peak 1).

In the first paper, describing the separation of plasmid DNA on monolithic disk columns, Giovaninni et al. [6] used

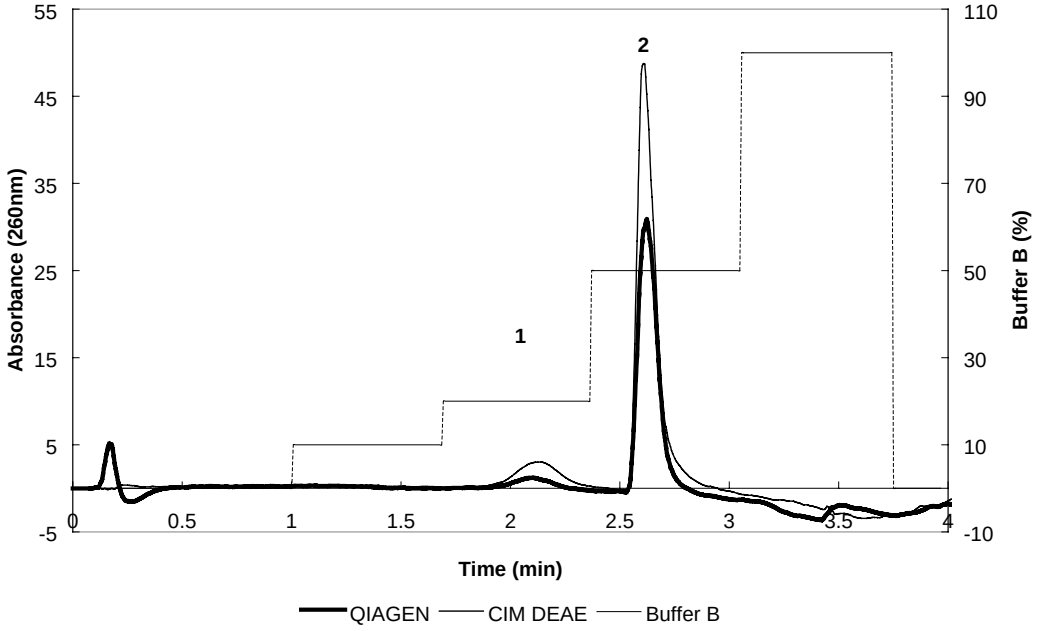

Fig. 5. Rechromatography of a purified plasmid pcDNA 3.1 on CIM® DEAE disk monolithic column and purified plasmid pcDNA 3.1 by QIAGEN kit on CIM® DEAE disk. Binding buffer: 25 mM MOPS, 0.5 M NaCl, pH 7.0, 15% isopropanol (v/v); elution buffer: 25 mM MOPS, 1.5 M NaCl, pH 7.0, 15% isopropanol (v/v); flow rate: 4 ml/min; stepwise gradient: 10, 20 and 50% steps (each step takes 0.68 min). (▬) absorbance at 260 nm for plasmid DNA purified by QIAGEN kit; (—) absorbance at 260 nm for plasmid DNA purified by monoliths; (···) increased concentration of salt. Peak 1: traces of RNA; peak 2: DNA.

plasmid DNA purified by a CsCl density gradient centrifugation in order to investigate the possibility for separating different isoforms of the molecule. In this paper, a possible use of monoliths for the purification (downstream processing) of plasmid DNA after alkaline lysis is shown. One of the main results emerging from this work is the fact, that a successful separation of plasmid DNA from cell RNA without any addition of exogenous RNase is feasible. Many current plasmid purification methods involve the addition of RNase, typically of bovine origin. In general, if the plasmids are used in the manufacture of pharmaceuticals it is desirable to avoid the addition of materials derived from animal especially bovine sources due to concerns regarding bovine sponigiform encephalopathies [32].

One of the confirmations for the successful isolation of plasmid DNA is an adequate homogeneity of at least 90% of covalently closed circular (ccc) form of plasmid DNA. As already mentioned, the purity of the obtained plasmid DNA was compared with the purity of plasmid DNA isolated by the QIAGEN commercial mini plasmid kit. Both purified plasmid DNAs (using commercial mini kit and using monolithic column), mainly contain the ccc form of plasmid (Fig. 2, lanes Q and 3). Apart from that, the isolated plasmid DNA is digested to completion by using different restriction enzymes. The quality of isolated plasmid DNA was also confirmed by transformation of *E. coli*. Namely, plasmid DNA isolated by using monolithic disk and by the QIAGEN kit has transformed *E. coli* with the same efficiency (data not shown). However, the purification on a DEAE monolithic disk was faster compared to purification with a QIAGEN kit. In addition, a regeneration of monoliths was already tested and results show the possibility of a consecutive usage of the same disk when the regeneration procedure is included after each run [30,31]. The $A_{260/A280}$ ratio in low-salt buffer is in the range of 1.8–2.0 thus confirming the purity of plasmid DNA. Other quality control tests like the levels of endotoxins, precise distribution of plasmid isoforms and capacity testing should be investigated in further experiments. With regard to the successful scale-up procedures for protein biotechnology using monoliths [33], similar results to the purification of plasmid DNA are expected.

4. Conclusion

Ongoing work shows that plasmid DNA can be isolated from bacterial cells using alkaline lysis followed by anion-exchange chromatography on a CIM® DEAE disk monolithic column. The purity of the isolated sample was similar when compared to established methods, but the separation was faster. Moreover, a successful separation of plasmid DNA from RNA without any addition of RNAse was obtained. As an additional advantage, a consecutive usage of the same CIM® DEAE monolithic column was possible due to a successful regeneration procedure. It was also shown that disk monolithic columns can be applied for fast analytical *in-process* control of the plasmid DNA purity.

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References

[1] D. Helinski, CRC Crit. Rev. Biochem. 7 (1979) 83.
[2] B.D. Davis, R. Dulbecco, H.H. Eisen, H.S. Ginsberg, Microbiology, third ed., Harper and Row, Philadelphia, PA, 1980.
[3] T. Schmidt, K. Friehs, M. Schleef, C. Voss, E. Flaschel, Anal. Biochem. 274 (1999) 235.
[4] J. Schorr, P. Moritz, M. Schleef, in: D.B. Lowrie, R.G. Whalen (Eds.), Methods in Molecular Medicine, DNA Vaccine: Methods and Protocols, vol. 29, Human Press Inc., Totawa, NJ, 1999, p. 11.
[5] A. Eon-Duval, K. Gumbs, C. Ellett, Biotechnol. Bioeng. 83 (2003) 544.
[6] R. Giovaninni, R. Freitag, T.B. Tennikova, Anal. Chem. 70 (1998) 3348.
[7] G.N.M. Ferreira, J.M.S. Cabral, D.M.F. Prazeres, Biotechnol. Prog. 16 (2000) 416.
[8] S. Lubbad, B. Mayr, C.G. Huber, M.R. Buchmeiser, J. Chromatogr. A 959 (2002) 121.
[9] M.M. Diogo, J.A. Queiroz, D.M.F. Prazeres, J. Chromatogr. A 998 (2003) 109.
[10] M.A. Winters, J.D. Richter, S.L. Sagar, A.L. Lee, R.J. Lander, Biotechnol. Prog. 19 (2003) 440.
[11] R. Lemmens, U. Olsson, T. Nyhammar, J. Stadler, J. Chromatogr. B 784 (2003) 291.
[12] T.B. Tennikova, B.G. Belenkii, F. Svec, J. Liq. Chromatogr. 13 (1990) 63.
[13] T. Tennikova, R. Freitag, in: H.Y. Aboul-Enein (Ed.), Analytical and Preparative Separation Methods of Biomacromolecules, Marcel Dekker, New York, 1999, pp. 255.
[14] M.A. Teeters, S.E. Conrady, B.L. Thomas, T.W. Root, E.N. Lightfoot, J. Chromatogr. A 989 (2003) 165.
[15] H.N. Endres, J.A.C. Johnson, C.A. Ross, J.K. Welp, M.R. Etzel, Biotechnol. Appl. Biochem. 37 (2003) 259.
[16] A. Strancar, P. Koselj, H. Schwinn, Dj. Josic, Anal. Chem. 68 (1996) 3483.
[17] J.L. Liao, in P.R. Brown, in: E. Grushka (Ed.), Advances in Chromatography, vol. 40, Marcel Dekker, New York, Basel, 2000, p. 467.
[18] K. Cabrera, D. Lubda, H. Eggenweiler, H. Minakichi, K. Nakanishi, J. High Resolut. Chromatogr. 23 (2000) 106.
[19] M. Schulte, D. Lubda, A. Delp, J. High. Resolut. Chromatogr. 23 (2000) 100.
[20] T.B. Tennikova, F. Svec, in: F. Svec, T.B. Tennikova, Z. Deyl (Eds.), Monolithic Materials in Journal of Chromatography Library, vol. 67, Elsevier, 2003, p. 352.
[21] A. Strancar, A. Podgornik, M. Barut, R. Necina, in: T. Scheper, R. Freitag (Eds.), Advances in Biochemical Engineering/Biotechnology, Vol. 76, Modern Advances in Chromatography, Springer–Verlag, Heidelberg, 2002, p. 49.
[22] A. Strancar, M. Barut, A. Podgornik, P. Koselj, Dj. Josic, A. Buchacher, LC–GC 10 (1998) 660.
[23] J. Sambrook, D.W. Russel, (Eds.), Molecular Cloning: A Laboratory Manual, second ed., Cold Spring Harbor Laboratory Press, 1989.
[24] H.C. Birnboim, Meth. Enzymol. 100 (1983) 243.
[25] QIAGEN Plasmid Mini Handbook, vol. 10, 2001.
[26] D.S. Holmes, M. Quigley, Anal. Biochem. 114 (1981) 193.

[27] H.C. Birnboim, J. Doly, Nucleic Acids Res. 7 (1979) 1513.
[28] A. Strancar, P. Koselj, H. Schwinn, Dj. Josic, Anal. Chem. 68 (1996) 3483.
[29] Dj. Josic, A. Strancar, Ind. Eng. Chem. Res. 38 (1999) 333.
[30] K. Branovic, A. Buchacher, M. Barut, A. Strancar, Dj. Josic, J. Chromatogr. A 903 (2000) 21.
[31] K. Branovic, G. Lattner, M. Barut, A. Strancar, Dj. Josic, A. Buchacher, J. Immun. Methods 271 (2002) 47.
[32] A.F. Hill, M. Desbruslais, S. Joiner, K.C. Sidle, I. Gowland, J. Collinge, L.J. Doey, P. Lantos, Nature 389 (1997) 448.
[33] A. Strancar, M. Barut, A. Podgornik, P. Koselj, H. Schwinn, P. Raspor, Dj. Josic, J. Chromatogr. A 760 (1997) 117.