Journal of Chromatography B, 877 (2009) 3257–3260

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Biorecognition of supercoiled plasmid DNA isoform in lysine-affinity chromatography

A. Sousa, F. Sousa*, J.A. Queiroz

CICS – Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, Av. Infante D. Henrique, 6201-001 Covilhã, Portugal

ARTICLE INFO

Article history:
Received 21 April 2009
Accepted 12 August 2009
Available online 21 August 2009

Keywords:
Affinity chromatography
Lysine support
Supercoiled plasmid DNA

ABSTRACT

The use of plasmid DNA-based therapeutics relies on procedures that efficiently purify the supercoiled plasmid isoform. The present study describes a new strategy that uses a lysine ligand in affinity chromatography to efficiently separate supercoiled and open circular plasmid DNA isoforms. To achieve higher specificity in this chromatography it is essential to characterize the behaviour of binding/elution of supercoiled isoforms. The results show that the lysine support promotes complex interactions with supercoiled isoform, according to ionic strength and temperature manipulation.

1. Introduction

The progress of disease-related genes and the possibility to manipulate the gene set-up in some organisms has fostered the development of innovative human DNA therapeutics, such as DNA vaccination, gene therapy and recombinant biopharmaceuticals [1]. In 2007, around 30% of gene therapy clinical tests started to use plasmids as non-viral vectors in vaccines for the treatment of cancer [2]. For the successful implementation of clinical approaches using plasmid DNA (pDNA)-based therapies, increasingly developments of production and purification procedures are required [3].

The biosynthesis of pDNA by *Escherichia coli* (*E. coli*) results in a highly enriched supercoiled (sc) pDNA extract, which is advantageous since sc pDNA is considered the most efficient isoform at transferring gene expression [4,5]. In this way, a particular downstream processing is required for the elimination of cellular components of the *E. coli* host, as well as for the reduction the open circular (oc), linear and denatured pDNA isoforms that occur due to conformational changes of sc plasmid. The main objective is the recovery of sc isoform (higher than 97%) according to the standards established by regulatory agencies [6].

One of the major bottlenecks found in the chromatography application for pDNA purification is that many stationary phases display poor separation selectivity towards pDNA and impurities due to their similar binding affinities [7]. The affinity chromatography (AC) takes advantage of the undoubted specificity and efficiency of pDNA or impurities to specific immobilized ligands [3,8,9]. These affinity interactions are probably the most adequate to purify sc pDNA, even when a single chromatographic step is required.

Supercoiled pDNA purification strategies that use amino acids (histidine and arginine) as immobilized ligands have recently lead to interesting results [10,11]. Particularly, immobilised histidine was used to specifically recognize sc pDNA present in the complex *E. coli* lysate, allowing the elimination of host impurities and the required purification degree [11]. In addition, arginine–agarose support has also been efficiently applied to separate plasmid isoforms, revealing a recognition of sc isoform [10]. Similarly to what is described for arginine, some molecular modelling studies described that lysine is a positively charged amino acid that mediates the largest number of contacts in protein–nucleic acid interactions [12,13]. Lysine–agarose was already used to separate RNA species of different molecular weight with a linear gradient of NaCl [14]. Building on these considerations, it is interesting to verify if the lysine matrix may contribute to the separation of sc and oc pDNA isoforms and to study the lysine-base molecular recognition mechanism by affinity chromatography.

2. Material and methods

Lysine-Sepharose 4B gel was obtained from GE Healthcare Biosciences (Uppsala, Sweden) and the Qiagen Plasmid Purification Maxi Kit was purchased from Qiagen (Hilden, Germany). Water was ultra-pure grade, purified with a Milli-Q system from Millipore. All salts used were of analytical grade.

* Corresponding author. Fax: +351 275 329 099.
E-mail address: fani.sousa@fcsaude.ubi.pt (F. Sousa).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.08.021

2.1. Bacterial production

The 6.05 kpb plasmid pVAX1-*LacZ* (Invitrogen, Carlsband, CA, USA) used in the experiments was produced by a cell culture of *Escherichia coli* DH5α. Growth was carried out at 37 °C using Terrific broth medium (20 g/l tryptone, 24 g/l yeast extract, 4 ml/l glycerol, 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4) supplemented with 30 μg/ml kanamycin. Growth was suspended at the late log phase [*OD*600 ~13] and the cells were recovered by centrifugation and were stored at −20 °C.

2.2. Lysis and isolation of plasmid DNA

pDNA was purified using the Qiagen (Hilden, Germany) plasmid maxi kit according to the manufacturer's instructions. The protocol is based on a modified alkaline lysis procedure. Following lysis, binding of pDNA to the Qiagen anion exchange resin is promoted under appropriate low-salt and pH conditions. Impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer and then is concentrated by isopropanol precipitation.

2.3. Preparative chromatography

Chromatography was performed in a Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences, Uppsala, Sweden). A 16 mm × 100 mm (~20-ml) column was packed with the commercial support Lysine-Sepharose 4B gel (GE Healthcare Biosciences, Uppsala, Sweden). This support is characterized by the manufacturer as a cross-linked 4% beaded agarose matrix with a 12-carbon atom spacer and a ligand density between 4 and 7 μmol/ml. Typically, the experiments were performed at 12 °C, through a circulating water bath to maintain the appropriate temperature. The column was equilibrated with 165 mM NaCl in 10 mM Tris buffer (pH 8.0) and the system was run at a flow rate of 1 ml/min. Plasmid sample (200 μl) was then injected at the same flow rate and the absorbance was monitored at 280 nm. After elution of unbound species with 165 mM NaCl in 10 mM Tris buffer (pH 8.0), the ionic strength of the buffer was increased stepwise to 250 mM NaCl in 10 mM Tris buffer (pH 8.0). Fractions were pooled according to the chromatograms obtained and kept for further analysis as described bellow. After chromatographic runs, it was necessary to wash the lysine medium with at least 5 bed volumes of 2 M NaCl in 10 mM Tris buffer, pH 8.0.

2.4. Gel electrophoresis

The fractions recovered from the chromatographic experiments were analysed by horizontal electrophoresis using 15-cm-long 1% agarose gels (Hoefer, San Francisco, CA, USA), which were stained with ethidium bromide (0.5 μg/ml). Electrophoresis was carried out at 100 V, for 1 h, with TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0).

3. Results and discussion

Initial experiments were performed to choose the best strategy for pDNA binding/elution, being observed that total (oc + sc) pDNA retention was obtained at 150 mM NaCl in 10 mM Tris buffer (pH 8.0), and total elution was verified at 250 mM with the same salt. During these experimental studies it is observed a need for strict control in order to maintain the reproducibility since a slight variation of the conductivity (salt concentration or temperature) affected the plasmid retention. The column was equilibrated with 165 mM NaCl in 10 mM Tris buffer (pH 8.0) at 12 °C, using a flow rate of 1 ml/min. Fig. 1 represents the pDNA chromatographic profile obtained when 200 μl (200 μg/ml) were injected. After plasmid injection, unbounded species were eluted due to lower affinity to the matrix. The ionic strength of the buffer was then increased to 250 mM NaCl in 10 mM Tris buffer (pH 8.0) and the highly bound species were eluted. Fractions were pooled according to the chromatograms obtained and were analysed by an agarose gel electrophoresis.

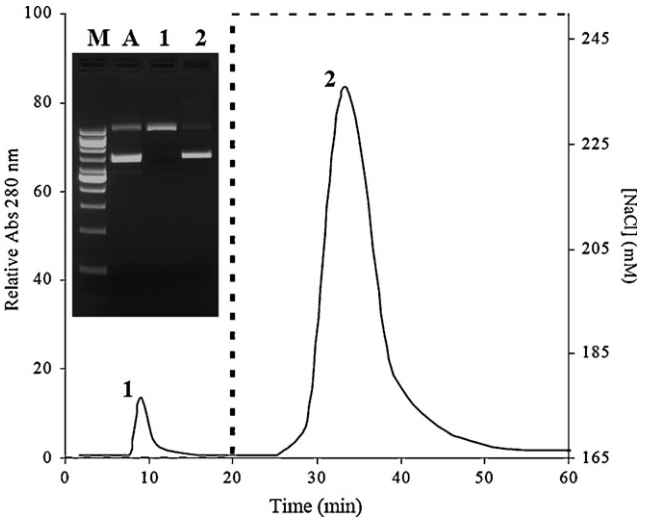

Fig. 1. Chromatographic separation of pDNA (200 μg/ml) isoforms with a lysine–agarose matrix. Elution was performed by stepwise increasing NaCl concentration in the eluent from 165 to 250 mM, as represented by dashed line. Agarose gel electrophoresis analysis of each peak is represented in the respective figure. Lane M: molecular weight marker; lane A: pDNA sample injected onto the column (oc + sc); lane 1: oc; lane 2: sc.

In Fig. 1, the analysis of electrophoresis gel revealed that the first peak of unbounded species corresponds to the oc isoform (lane 1), and the second peak was attributed to the sc isoform (lane 2). As judged by the gel, both isoforms were totally isolated. These results suggest that the recognition of lysine matrix by sc isoform can be related with supercoiling phenomenon that is a consequence of deformations induced by the torsional strain leading to a higher exposition of the bases of sc isoform compared with the oc isoform. This phenomenon can be considered as an explanation for the fact of lysine ligand to distinguish and differentially interact with both isoforms (Fig. 1) further suggesting a specific recognition of the sc pDNA isoform.

Although lysine can only use a single side chain atom for binding, and the hydrogen bonds are less likely to resemble the ideal geometry than for arginine [3], there are clear evidences that lysine is a multiple donor amino acid and promotes hydrogen complex interactions (bidentate) preferentially with guanine base, according to what was found in molecular modelling studies [12]. Even though the salt concentrations (250 mM NaCl) needed to elute bound plasmid isoforms from lysine matrix (Fig. 1) are lower than those typically used in other anion-change chromatographic supports (higher than 500 mM), the presence of electrostatic interactions between the plasmid phosphate groups and lysine ligand (positively charged) should be considered, similarly to what happens in studies with arginine [5].

To better understand the mechanism for specific recognition of sc pDNA with lysine–agarose, some experiments of competitive elution with elution buffers containing different kinds of amino acids were performed. A 40 min linear gradient between 10 mM Tris buffer and 250 mM of each amino acid was used. Arginine was chosen as a positively amino acid in the elution buffer and pDNA was eluted during the linear gradient. This behaviour is due to the

positively character of this amino acid that promote a preferential binding of pDNA with free arginine present in the buffer by electrostatic interactions, inducing its elution together with arginine. When it was used glutamic acid as negative amino acid in the elution buffer, pDNA was also eluted because glutamic acid promoted the displacement of the bound pDNA. So, it was observed that the use of a negative amino acid can also promote a competitive elution, competing with pDNA, particularly with the phosphate groups, for the lysine ligands surface. Valine was chosen as amino acid with aliphatic side chain, and for this case, the elution gradient did not induce pDNA elution. This fact shows that the preferential interaction between pDNA and lysine support is ionic and not hydrophobic, because despite this amino acid have a carbon lateral chain, pDNA remained bound at the matrix (results not shown). These data allowed the knowledge of the underlying mechanism and the responsible groups for the recognition of pDNA by lysine, which can be extremely helpful to the further efforts in development of novel materials for purification of nucleic acids.

To further confirm the matrix selectivity at the same elution conditions, one sample with sc isoform (200 μg/ml) obtained after lysis and purification with the Qiagen kit, and another sample with oc isoform (200 μg/ml) achieved by incubation of a sc pDNA sample at room temperature for 5 days (following by electrophoresis gel), were used (results not shown).

The effect of temperature on selectivity was already described in other studies and it has been proved that the temperature play an important role in pDNA affinity chromatography with amino acids ligands. In the histidine matrix, the temperature increase only affected the nucleic acids molecules conformation. Some studies described that the temperature increase induces plasmids torsional strain changes leading to relaxation and consequent decrease in the interaction of sc pDNA with the histidine ligands. However, the study of single-strand molecules revealed that the increase in temperature induced a greater exposure of hydrophobic contact area of these molecules leading to an increase in their retention [15]. On the other hand, in arginine chromatography, the pDNA molecules retention is directly influenced by temperature, suggesting that hydrophobic interactions may be involved [10,16]. In the study of separation of ribonucleic acids on lysine–agarose, the decrease of temperature promotes a greater retention of the tRNA species to the column [14]. Therefore, several chromatographic analysis were triggered to demonstrate how temperature influences the retention of pDNA isoforms on the lysine support. A linear gradient (3 column volumes) between 160 and 250 mM NaCl Tris buffer (pH 8.0) was used, and to stabilize the required temperature (5, 12, 15 and 24 °C) for each experiment, a circular water bath was connected to the column. Fig. 2 represents the elution profiles obtained when loading a pDNA sample containing oc and sc pDNA isoforms into the lysine matrix. It was evidenced that, while the temperature of the column decreases, also the sc isoform retention decreases, being partially eluted in the first peak together with the oc isoform. This tendency can be monitored by the different height of peaks and

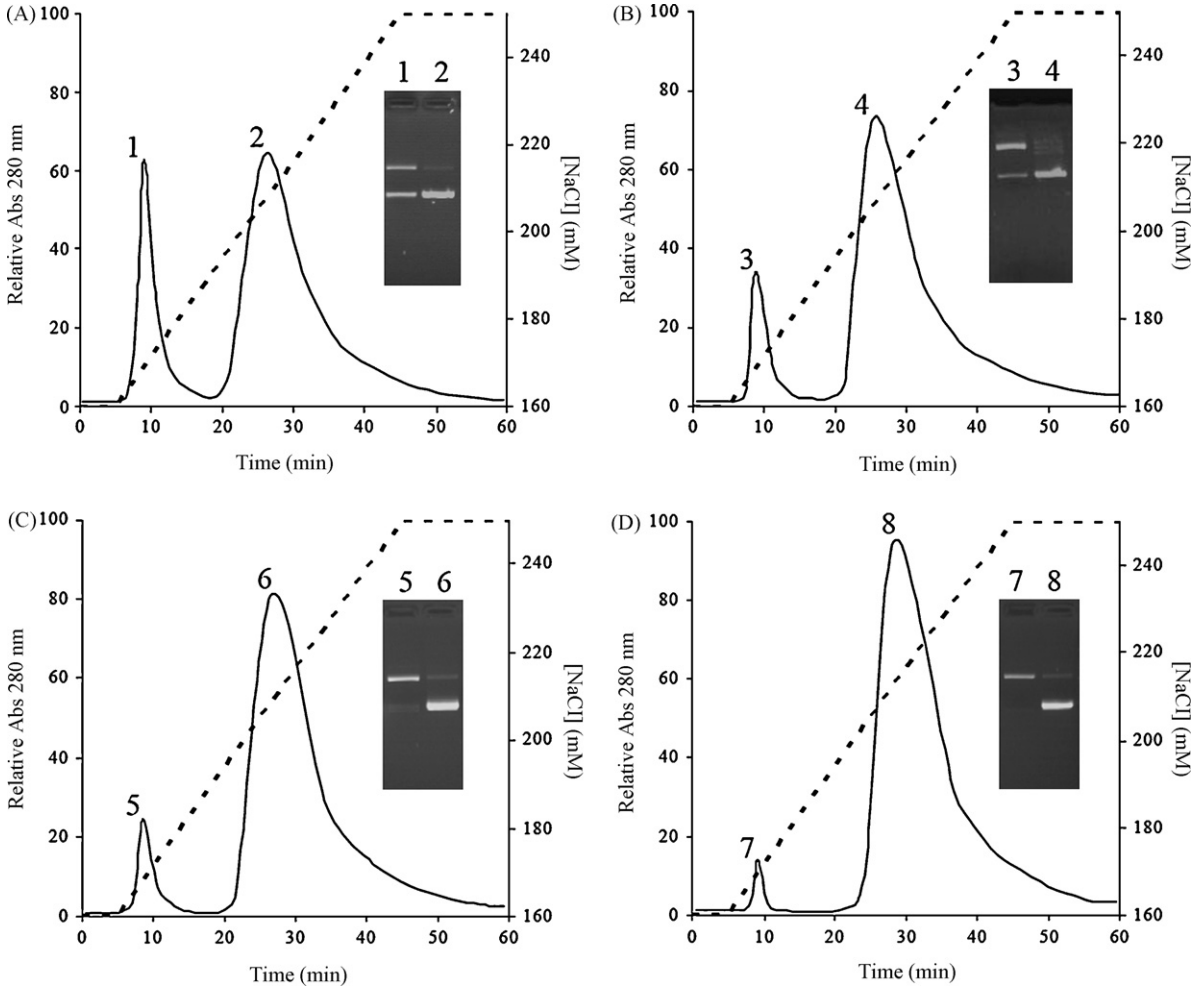

Fig. 2. Affinity chromatographic profiles of oc and sc pDNA isoforms retention on lysine–agarose support, at different temperatures: (A) 5 °C; (B) 12 °C; (C) 15 °C and (D) 24 °C. Elution was performed at 1 ml/min by linearly increasing the NaCl concentration in the eluent from 160 to 250 mM (3 column volumes). Agarose gel electrophoresis analysis of each peak is represented in each chromatogram.

by electrophoresis gel presented in each chromatogram (Fig. 2). The fact of the retention of sc isoform increased with increasing temperature may be related to the presence of hydrophobic interactions with the aliphatic portion of side chain or the 12-carbon epoxy spacer arm of lysine ligand [7].

Curiously, in this work it is visible that the first peak of each chromatogram was eluted approximately at same retention time, as well as happens with the second peak. However, in arginine study [10], the two peaks tend to approach and the two isoforms are more retained due to the temperature increase. These different behaviours suggest that, in case of lysine chromatography, the temperature increase allows stronger sc isoform retention, and this interaction at low temperatures is not so favoured. Thus, it is evident that temperatures above 12 °C promote a significant effect in sc isoform distribution, increasing the specificity of the retention and favouring the total plasmid isoforms separation. As previously demonstrated by the competitive elution studies, the hydrophobic interactions are not the preferential forces involved in sc plasmid retention, and as it is known a temperature increase significantly weaken nonspecific interactions like hydrogen bonds. Hence, the behaviour found with the temperature increase suggests that the ionic interaction between lysine and pDNA is strengthened favouring the recognition of sc isoform.

In general, some positively charged amino acids were already used as affinity ligands to efficiently separate sc and oc pDNA isoforms, but each matrix can specifically recognize and differently interact with the sc isoform. In the case of histidine-based affinity matrix it was achieved the possibility to separate the pDNA isoforms using a high ammonium sulphate concentration. This fact can be considered a disadvantage indicating the need for further improvements. It also verified that the temperature had only a direct influence on molecules conformational structure [15]. Otherwise, the arginine matrix revealed an extreme sensitivity of the binding/elution behaviour of the biomolecules with respect to the ionic strength of the buffers used. The type of interactions established between the arginine ligand and the nucleic acid molecules were different. Thus, the molecules retention was performed at low sodium chloride concentrations and their elution was obtained with a slight increase of salt concentration [10]. In the arginine support studies, the temperature effect was more evident on the retention [16]. In the present study, plasmid isoforms were also separated using mild salt conditions and it is suspected the involvement of different interactions. As it was previously described, in lysine chromatography, the temperature increase developed an important role allowing a specific interaction with the sc isoform. Interestingly, unlike arginine, the retention time of oc and sc isoforms in lysine matrix was not changed with the temperature increase, but an increased specificity was observed favouring the total plasmid isoforms separation.

For the first time, it is presented a new affinity chromatographic process based on naturally occurring interactions, between the lysine amino acid and plasmid molecules, promoting an efficient separation of sc and oc isoforms. We suggest that the underlying mechanism involves phenomenological interactions like biorecognition between the lysine matrix and pDNA isoforms, including hydrogen, electrostatic, van der Waals and hydrophobic interactions. Lysine-affinity chromatography can be another potential purification technique to obtain the sc pDNA directly from a clarified *E. coli* lysate in the required conditions for therapeutic applications, since in this work it was proved that this matrix recognize specifically the sc isoform. The use of amino acid matrices and mild salt conditions become this technique more appropriate and economic to apply at biotechnological process and industrial scale.

Acknowledgements

This work was supported by FCT, the Portuguese Foundation for Science and Technology (PTDC/EQU-EQU/65492/2006), A. Sousa also acknowledges a fellowship (SFRH/BD/41390/2007) from FCT.

References

[1] M. Schleef, T. Schmidt, J. Gene Med. 6 (Suppl. 1) (2004) S45.
[2] R.J. Anderson, J. Schneider 25 (Suppl. 2) (2007) B24.
[3] F. Sousa, D.M.F. Prazeres, J.A. Queiroz, Trends Biotechnol. 26 (9) (2008) 518.
[4] L. Cupillard, V. Juillard, S. Latour, G. Colombet, N. Cachet, S. Richard, S. Blanchard, L. Fischer. 23 (2005) 1910.
[5] F. Sousa, D.M.F. Prazeres, J.A. Queiroz, J. Gene Med. 11 (1) (2009) 79.
[6] J. Stadler, R. Lemmens, T. Nyhammar, J. Gene Med. 6 (Suppl 1) (2004) S54.
[7] M.M. Diogo, J.A. Queiroz, D.M.F. Prazeres, J. Chromatogr. A 1069 (2005) 3.
[8] J.C. Murphy, D.L. Jewell, K.I. White, G.E. Fox, R.C. Willson, Biotechnol. Prog. 19 (2003) 982.
[9] Y. Han, G.M. Forde, J. Chromatogr. B 874 (2008) 21.
[10] F. Sousa, T. Matos, D.M.F. Prazeres, J.A. Queiroz, Anal. Biochem. 374 (2008) 432.
[11] F. Sousa, C.T. Tomaz, D.M. Prazeres, J.A. Queiroz, Anal. Biochem. 343 (2005) 183.
[12] N.M. Luscombe, R.A. Laskowski, J.M. Thornton, Nucleic Acids Res. 29 (2001) 2860.
[13] M.M. Hoffman, M.A. Khrapov, J.C. Cox, J. Yao, L. Tong, A.D. Ellington, Nucleic Acids Res. 32 (2004) D174.
[14] D.S. Jones, H.K. Lundgren, F.T. Jay, Nucleic Acids Res. 3 (1976) 1569.
[15] A. Sousa, F. Sousa, D.M.F. Prazeres, J.A. Queiroz, Biomed. Chromatogr. 23 (2009) 745.
[16] A. Sousa, F. Sousa, J.A. Queiroz, J. Sep. Sci. 32 (2009) 1665.