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Performance of a non-grafted monolithic support for purification of supercoiled plasmid DNA ☆

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ABSTRACT

The use of therapeutics based on plasmid DNA (pDNA) relies on procedures that efficiently produce and purify the supercoiled (sc) plasmid isoform. Several chromatographic methods have been applied for the sc plasmid purification, but with most of them it is not possible to obtain the required purity degree and the majority of the supports used present low capacity to bind the plasmid molecules. However, the chromatographic monolithic supports are an interesting alternative to conventional supports due to their excellent mass transfer properties and their high binding capacity for pDNA. The separation of pDNA isoforms, using short non-grafted monolithic column with CarbonylDiImidazole (CDI) functional groups, is described in the current work. The effect of different flow rates on plasmid isoforms separation was also verified. Several breakthrough experiments were designed to study the effect of different parameters such as pDNA topology and concentration as well as flow rate on the monolithic support binding capacity. One of the most striking results is related to the specific recognition of the sc isoform by this CDI monolith, without flow rate dependence. Additionally, the binding capacity has been found to be significantly higher for sc plasmid, probably because of its compact structure, being also improved when using feedstock with increased plasmid concentrations and decreased linear velocity. In fact, this new monolithic support arises as a powerful instrument on the sc pDNA purification for further clinical applications.

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1. Introduction

The requirements in medicine and molecular biology science, demand large quantities of highly pure and homogenous plasmid DNA (pDNA) for different applications such as cloning, large scale protein production, DNA vaccination or gene therapy [1]. The impact of plasmid structure and conformation on the transfection process efficacy has received some attention in the past. Thus, the supercoiled (sc) pDNA isoform is the desired topological form since it induces the most efficient access to the nucleus of the cell and consequently improves gene expression in eukaryotic cells [2]. According to international regulations, the product-quality is defined as a percentage of the sc isoform compared to the total pDNA, being that the purity of the sc pDNA must be 100% with the homogeneity degree higher than 97% of sc isoform from *Escherichia coli* (*E. coli*) host [3]. This foreseeable necessity led to amplified efforts within both research and industry to improve the effectiveness of sc pDNA production and purification methodologies [4].

For the purification of pDNA several chromatographic methods based on particulate supports have been reported. Besides conventional techniques such as anion exchange, hydrophobic interaction and size exclusion chromatography [5], other methods were tested with more or less success. The affinity concept has gained impact with the development of new supports combining different and more specific interactions with pDNA. Based on the natural occurrence of protein–DNA complexes in biological systems, and the atomic studies demonstrating the existence of favored interactions between particular amino acids and nucleic acid bases [6,7], it was recently developed by our research group a new affinity chromatographic methodology using some amino acids immobilized on agarose matrix for the isolation and purification of several nucleic acids [8]. Particularly, histidine [9], arginine [4] and lysine [10] were already used as ligands to efficiently purify sc pDNA, and the recognition of this isoform proved the presence of specific interactions between pDNA molecule and the amino acid-based matrices studied. Despite these results obtained with the affinity matrices, some problems remained to be solved, such as the low capacity of available supports for pDNA and the low diffusivity of pDNA samples due to their high molecular weight. Considering these facts, it is necessary to study and develop more suitable supports to overcome these problems.

Monolithic sorbents represent one of the newest developments of chromatographic stationary phases for biomolecules separation

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and purification. A typical monolith is a continuous bed constituted by a single piece of highly porous solid material, whose pore size depends on the polymerization process [11,12]. The ligands immobilized on these chromatographic supports can be several biological agents as antibodies, enzymes, lectins or amino acids [13] and peptides.

The chromatographic support named as Convective Interaction Media (CIM) was developed with the aim of obtaining a short chromatographic layer, ensuring well-defined, narrow pore-size distributions, excellent separation power and exceptional chemical stability [11]. These innovative CIM disks present numerous advantages comparing to conventional supports applied on sc pDNA purification, among which it is important to refer: high binding capacity due to excellent mass transfer properties and a huge quantity of accessible binding sites for large biomolecules as pDNA [14]; capacity to achieve very fast separation and purification with high reproducibility both at small and large scale [15]; simple handling, flow independent resolution [16]; the target molecule can be eluted in a concentrated form with a reduced biomolecule degradation due to short contact times with the chromatographic matrix [17].

This novel chromatographic material has been largely employed to separate immunoglobulins [18], oligonucleotides [19], proteins [19,20] and recently to purify pDNA [14,21–23]. Knowing that an agarose based matrix with immobilized histidine amino acid allows an efficient separation of pDNA isoforms [9], it becomes interesting to study a new non-grafted glycidyl methacrylate monolith (BIA Separations, Ljubljana, Slovenia) since this support is constituted by CarbonylDiImidazole (CDI) chemical groups. Pointing to the possibility of specific recognition mechanisms between imidazole ring and pDNA molecules, the applicability of CDI monolithic support to purify sc isoform seems to present great potential and is exploited in the present work. Additional chromatographic characterization based on breakthrough experiments was also designed to study the effect of parameters such as, pDNA topology and concentration, as well as the different flow-rate on the monolith dynamic binding capacity.

2. Experimental

2.1. Materials

All experiments were carried out in a 0.34 mL (3 mm thick and 12 mm diameter) non-grafted CDI poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolith packed into a CIM disk housing, provided by BIA Separations (Ljubljana, Slovenia). This CIM epoxy monolith was initially synthesized from glycidyl methacrylate and ethylene dimethacrylate monomers in presence of porogens dodecanol and cyclohexanol by BIA Separations Company. The next step consisted in hydrolyzing the epoxy monolith with 0.5 M H_2SO_4 at 60 °C for 2–3 h to obtain hydroxyl groups. Finally, the hydroxyl groups were treated with 1,1′-carbonyldiimidazole to originate the imidazole carbamate reactive groups, as it was described by Bencina et al. [24]. Qiagen Plasmid Purification Maxi Kit was from Qiagen (Hilden, Germany). The ammonium sulfate used in the elution buffers was purchased from Panreac (Barcelona, Spain). All solutions were freshly prepared using deionized water ultra-pure grade, purified with a Milli-Q system from Millipore (Billerica, MA, USA) and analytical grade reagents. The elution buffers were filtered through a 0.20 μm pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically.

2.2. Methods

2.2.1. Bacterial cell culture

The 6.05-kbp plasmid pVAX1-*LacZ* (Invitrogen, Carlsband, CA, USA) used in the experiments was produced by a cell culture of *E. coli* DH5α, being obtained a high plasmid copy numbers enriched in sc isoform. 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 ($OD_{600} \approx 13$) and the cells were recovered by centrifugation and were stored at −20 °C.

2.2.2. Alkaline cell lysis and pDNA isolation

Plasmid DNA was isolated from *E. coli* bacteria after alkaline lysis using the Qiagen (Hilden, Germany) plasmid maxi kit according to the manufacturer's instructions. The protocol is based on a modified alkaline lysis procedure, being considered a critical unit operation, because this step can influence the final ratio of sc plasmid. Unfavorable environmental conditions, such as extreme pH and temperature, affect the helical repeat of DNA promoting its unwinding, being generated other forms such as open circular (oc) isoform. 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, and pDNA is eluted in a high-salt buffer, being then concentrated through an isopropanol precipitation. The pDNA obtained at the end of alkaline lysis contained around 90% of sc isoform, while the remaining 10% are due to the presence of oc isoform, as revealed by agarose electrophoresis. This pDNA isoform was applied for preparative chromatography studies and dynamic binding capacity measurements. Open circular (oc) pDNA, also used for dynamic binding capacity experiments, was prepared by incubating a sc pDNA sample at room temperature (24 °C). The sample was monitored over the time by electrophoresis analysis until the total conversion of sc plasmid to oc isoform was observed (about 3 days).

2.2.3. Preparative chromatography

All experiments were performed using an ÄKTA Purifier system (GE Healthcare Biosciences, Uppsala, Sweden) consisting of a compact separation unit and a personal computer with Unicorn control system Version 5.11. The monolithic column was equilibrated with 2.3 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 8.0) and the system was run at a flow rate of 132.5 cm/h. Plasmid sample, resultant from alkaline lysis and pre-purification with the Qiagen Kit, was homogenized in 50 mM phosphate buffer (pH 8.0) and the ionic strength was corrected, dissolving the ammonium sulfate quantity required for the sample volume that will be injected (loop with 20 μL). The absorbance was monitored at 260 nm. After elution of unbound species with 2.3 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 8.0), the ionic strength of the buffer was decreased by shifting the eluting buffer to 0 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 8.0) to elute bound species. Following plasmid isoforms separation with an appropriate elution gradient, as described above, the fractions were pooled according to the chromatograms obtained, and used for further electrophoresis analysis after concentration and desalting with Vivaspin concentrators (Vivascience). A posterior study was conducted to analyze the effect of different flow rates (53, 132.5, 265 and 397.5 cm/h) on the resolution of both plasmid isoforms. All experiments were performed at room temperature.

2.2.4. Dynamic binding capacity (DBC) measurement for pDNA

The monolithic column, with non-grafted CDI-disk previously applied in preparative chromatography, was used for the determination of the dynamic binding capacity for pDNA. These experiments were conducted using different pDNA topologies (sc and oc) and concentrations (0.05, 0.1, 0.15 and 0.2 mg/mL), as well as different flow rates (26.5 and 53 cm/h). The column was equilibrated with 2.5 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 8.0), and it was necessary to prepare the feedstock with high plasmid quantities to test the parameters referred above. After several alka-

line lysis processes using the Qiagen Kit, the final plasmid product was homogenized in 50 mM phosphate buffer (around 100 mL) and the ionic strength was corrected, dissolving the ammonium sulfate necessary, but in this case for a concentration of 2.5 M. The binding capacity can be measured by different methods [25]. In this work, it was applied the open-loop frontal analysis that is similar to closed-loop frontal analysis, but the feedstock solution is collected after crossing the whole system instead of being transported back into the closed circuit. At the end of each experiment, the binding capacity was determined by the breakthrough area integration method [26]. Briefly, each breakthrough experiment was derived from a 100% of saturated column. Then the sample volume corresponding to the adsorbed amount of plasmid was calculated by numerical integration of the detector response. The area obtained from the filled column was subtracted from that for the empty column. In this step, the void volume was correctly discounted from the DBC determination. This area is equivalent to the sample volume, which was required to saturate the column, and can be related with sample concentration that remained bound per millilitre of the support, reflecting the support capacity. Normally, the capacity values are represented at 10% of the breakthrough that corresponds to 10% of the column saturation, being calculated in the same way. Finally, the elution of the bound plasmid was achieved by decreasing the ammonium sulfate concentration in the mobile phase to 0 M in a stepwise manner.

2.2.5. Gel electrophoresis

The fractions recovered from each chromatographic experiment, after being efficiently desalinized, were analyzed by horizontal electrophoresis using 15-cm-long 1% agarose gels (Hoefer, San Francisco, CA, USA), stained with ethidium bromide (0.5 μg/mL). Electrophoresis was carried out at 100 V, for 40 min, with TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0).

3. Results and discussion

CIM supports represent the fourth generation of monoliths chromatographic supports. These glycidyl methacrylate-based monolithic columns are characterized by a single piece that contains pores highly interconnected, forming a network of channels [15]. Thus, the whole mobile phase is forced to run through these pores duo to the pressure difference, therefore the mass transfer between stationary and mobile phase is based on convection rather than on diffusion, increasing their mobility by several orders of magnitude. This transport mechanism enables very fast separations and purification of components as well as flow-unaffected resolution and dynamic binding capacity [15]. The latter is especially important for the purification of molecules on preparative level where the productivity is essential. Besides their attractive hydrodynamic characteristics, the non-grafted CDI monolith, provided by BIA Separations (Ljubljana, Slovenia) becomes a potential chromatographic support for pDNA purification.

3.1. Supercoiled pDNA purification

Initial experiments were performed to choose the best strategy to achieve the binding and elution of the pDNA isoforms, being observed that the total (oc + sc) pDNA elution was obtained at 1 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 8.0). However, the partial retention of sc isoform was established at 2 M and total retention of both isoforms was verified at 2.5 M with the same salt (data not shown). These results suggest that it is necessary to apply high ammonium sulfate concentrations on the binding buffer and to optimize the concentration range between 2.5 and 2 M to obtain the ideal plasmid isoforms separation.

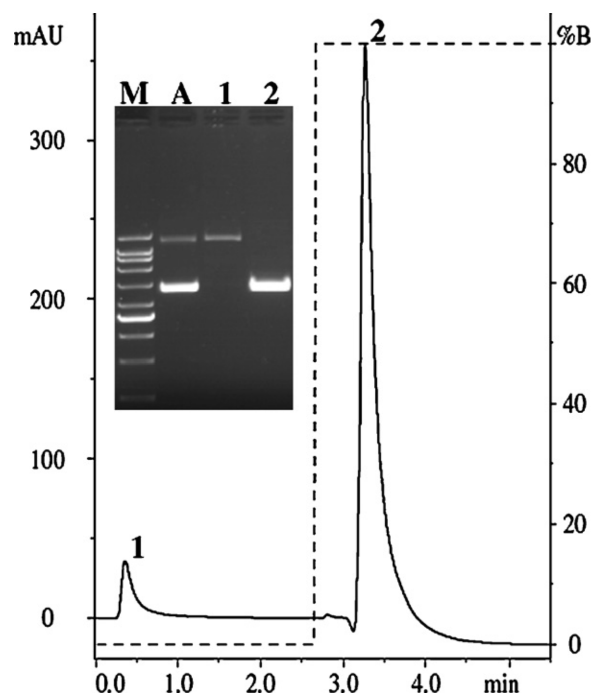

Fig. 1. Chromatographic purification of sc isoform pDNA using a non-grafted CDI monolith. Mobile phase – buffer A: 2.3 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer pH 8.0; buffer B: 50 mM phosphate buffer pH 8.0. Step elution was performed at 132.5 cm/h (2.5 mL/min) by increasing the buffer B percentage in the eluent from 0% to 100% of buffer B, as represented by the dashed line. UV detection at 260 nm. Injection volume was 20 μL. Agarose gel electrophoresis analysis of each peak is represented in respective chromatogram. Lane M: molecular weight marker; lane A: pDNA sample injected onto the column (oc + sc); lane 1: oc; lane 2: sc.

The monolithic column was equilibrated with 2.3 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 8.0) using a flow rate of 132.5 cm/h. After native plasmid DNA (oc and sc isoforms) sample injection and binding to the column, a first elution step designed to elute the species with lower affinity to the matrix was carried out with the same salt concentration of the equilibrium buffer. The elution of highly bound species was then performed with a second gradient step by decreasing to 0 M $(NH_4)_2SO_4$ (in 50 mM phosphate buffer, pH 8.0). The agarose gel electrophoresis analysis of the fractions eluting from the column (Fig. 1) proved that the first peak of unbound material corresponds to the oc isoform (lane 1), whereas the second peak was attributed to the sc isoform (lane 2). As judged by the gel, both isoforms were totally isolated and sc isoform appears completely purified. The fact of carbonyldiimidazole groups of monolith differentially interact with both plasmid isoforms at high concentration of ammonium sulfate (Fig. 1), evidenced by a stronger retention of sc isoform, can be tentatively explained by the supercoiling phenomenon. Hypothetical interactions of the CDI monolith with the pDNA backbone can be ruled out because phosphate and sugar groups are equally exposed in both isoforms. Under these ionic strength conditions the isoforms retention is mostly due to the hydrophobic interactions. Nevertheless, the observed selectivity between these different isoforms must be due to interactions of the imidazol ring with the bases of sc pDNA but not with the bases of oc pDNA. The reason for this behavior is the deformation induced by torsional strain in the sc isoform that becomes the bases of this nucleic acid more exposed than the bases of the oc isoform. Previous works related with affinity chromatography have revealed the same preference by this isoform [9,10]. Curiously, when the histidine–agarose matrix was applied to purify the sc isoform [9], the salt and ionic strength conditions used were the same to those used in the present work. Consid-

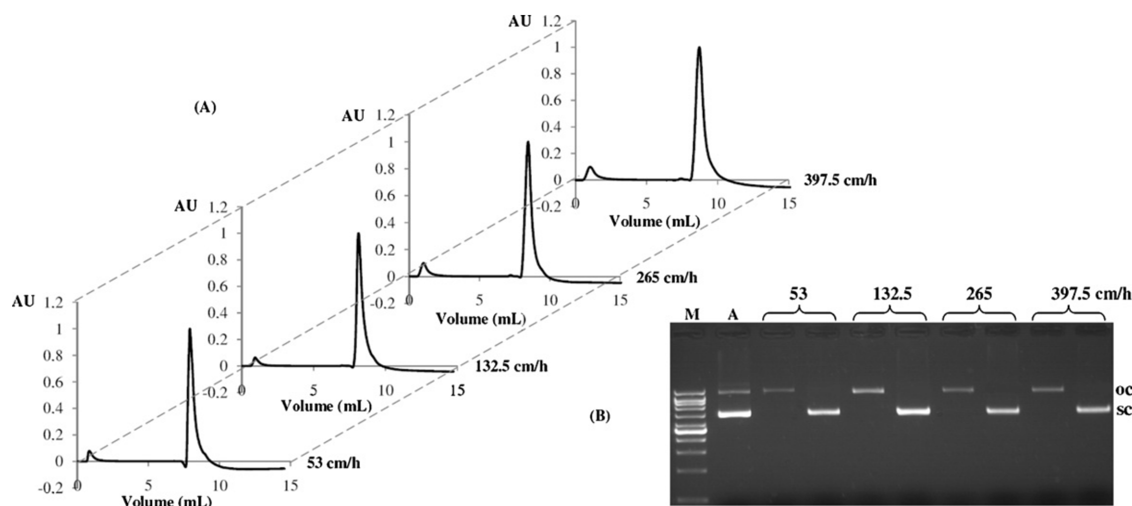

Fig. 2. (A) Effect of flow rate on resolution of pDNA isoforms. Experiments were performed in the same salt and gradient conditions applied for the sc isoform purification (presented in Fig. 1) at different flow rates (53, 132.5, 265 and 397.5 cm/h). (B) The peaks obtained in the chromatographic runs at different flow rates were identified by agarose gel electrophoresis. Lane M: molecular weight marker; lane A: pDNA sample injected onto the column (oc + sc).

ering that histidine matrix is composed by an epoxy spacer arm connected to a histidine amino acid, which is also constituted by an imidazole ring, it can be suggested that the recognition of sc isoform by both matrices, histidine–agarose and CDI–monolith, is mainly related with imidazole ring interactions. As it was described on histidine study [9], the interactions responsible for this specific recognition are mostly hydrophobic since high ammonium sulfate concentrations were used. Nevertheless, other elementary interaction forces that constitute the affinity interactions are also involved, such as (bidentate) H-bond interactions between the H donor and the H acceptor atoms in the nonprotonated imidazol ring with base edges, ring stacking/hydrophobic interactions, and water-mediated H bonds, that allow a stronger binding with sc plasmid bases.

3.2. Effect of flow rate on plasmid isoforms separation

Considering the physical and chemical constitution of methacrylate monolithic columns, high flow rates are expected to be used for semi-preparative or preparative purification of biomolecules as pDNA [16], and no change is expected on the separation selectivity. In fact, some studies proved that the molecules separation is flow-independent due to the monoliths characteristics [15,16]. Therefore, to verify the impact of flow rate on resolution of isoforms, the same elution gradient previously established for the ideal separation was used. Fig. 2(A) shows the resulting chromatograms for the separation of pDNA isoforms at the different flow rates; 53, 132.5, 265, and 397.5 cm/h. All chromatograms were normalized in function of the elution volume that was the same for all experiments performed, but a significant reduction on the chromatographic run time was verified when higher flow rates were used. The purity of each plasmid isoform separated with different flow rates was followed by electrophoresis as shown in Fig. 2(B). Evaluating the chromatograms and the respective electrophoresis, it is clearly evident that no changes have occurred in the separation efficiency of plasmid isoforms for the different flow rates under study. It is also possible to observe that the chromatograms overlap each other even at the highest applied flow rate of 397.5 cm/h. In this case the separation was completed in 2 min. This result is in accordance with other studies employing anion-exchange monoliths for pDNA purification where it was also verified good peaks resolution even at increased linear velocities [14].

3.3. Dynamic binding capacity determination

3.3.1. Effect of pDNA topology

The determination of column binding capacity is a critical component of the purification process development. Monoliths are known as versatile matrices that present good structural characteristics responsible for their high dynamic binding capacities and flow-unaffected resolution, when compared for instance with alternative agarose-based supports. In the present study, the influence of pDNA topology (oc and sc isoforms) on the binding capacity was evaluated. Breakthrough experiments were performed using feedstock solutions with the respective pDNA isoform at 0.05 mg/mL (Fig. 3). The feedstock used to study the effect of sc plasmid topology on capacity was obtained immediately after alkaline lysis and prepurification with the Qiagen Kit. Although most of plasmid obtained presents sc conformation, a residual quantity of this isoform is converted into oc form, being obtained a solution with around 90% of sc isoform and 10% of oc isoform. On the other hand, when it

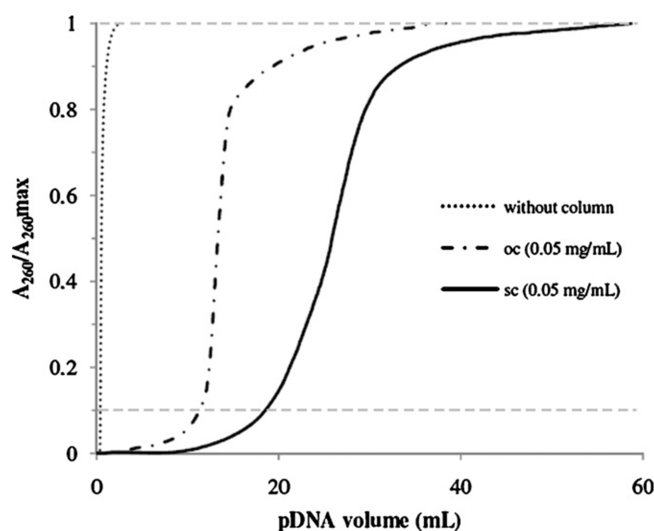

Fig. 3. Influence of plasmid topology on normalized breakthrough curves of CDI monolith. Representation of breakthrough experiments without column, open circular and supercoiled conformations. Flow rate: 53 cm/h (1 mL/min); feedstock: sc or oc plasmid solutions, at 0.05 mg/mL, were prepared in 2.5 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer pH 8.0.

Table 1
Effect of sc plasmid concentration on total and 10% dynamic binding capacity of non-grafted CDI monolith. The breakthrough experiments were performed on a single monolithic disk with 0.34 mL, at flow rate of 53 cm/h (1 mL/min). The loading was constituted by sc isoform dissolved in 2.5 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer, pH 8.0.

Plasmid concentration (mg/mL)	DBC ± SD, $n = 2$	
	10%[a]	Total[b]
0.05	2.193 ± 0.003	3.640 ± 0.005
0.10	2.206 ± 0.004	3.900 ± 0.011
0.15	2.215 ± 0.003	4.412 ± 0.006
0.20	3.380 ± 0.006	6.359 ± 0.008

[a] Dynamic binding capacity at 10% breakthrough (mg/mL).
[b] Total dynamic binding capacity (mg/mL).

was studied the effect of oc conformation, sc form was converted to oc isoform by incubation at room temperature (24 °C). For this case, it was considered that the feedstock had almost 100% of oc isoform because after 3 days only this isoform appeared on the electrophoresis analysis. A significant difference in the binding capacity curves was observed for the two pDNA conformations, and the lower capacity was obtained with oc pDNA solutions. This behavior can be explained by the high supercoiled degree of sc isoform that enables the reduction of the superficial contact area, facilitating the binding of other biomolecules and improving the capacity. Thus, in this work, the breakthrough curve of oc isoform presents less capacity than sc isoform, probably because oc molecule has larger apparent size. These results strengthen the theory that the apparent size or conformation of the pDNA plays an important role in binding to chromatographic supports [27,28].

Analyzing the profile of the curves obtained in Fig. 3 it is observed that instead of the curves sharp increase, they present a typical S shape and a gradual creep up until the column was saturated. The profile obtained for both breakthrough curves of pDNA isoforms is also in agreement with the work of Mihelic and collaborators, who found out that some factors can lead to appearance of S shape curves and to the extension of the time required for equilibration [25]. These factors that affect and can decrease the dynamic binding capacity of monolithic matrices can be the increasing flow rates, solute size and high viscosity of mobile phases. Moreover, particular studies revealed that the increased capacity can be presumably associated to biomolecules agglomeration, also having a pronounced influence on the tailing of breakthrough curves, when it is applied the open-loop frontal analysis [29]. Thus, the higher dynamic binding capacity found for the sc pDNA isoform can also be related with the higher compactness of this biomolecule.

3.3.2. Effect of pDNA concentration

Since it was demonstrated that the feedstock of sc pDNA isoform allowed a higher capacity value, it is also interesting to verify whether different concentrations of the feedstock affect the CDI monolith capacity. For this purpose, several breakthrough experiments were performed using different pDNA concentrations (0.05, 0.1, 0.15 and 0.2 mg/mL), with the flow rate of 53 cm/h. The results presented in Table 1 show that dynamic binding capacity can be improved by increasing the sc pDNA concentration in the feedstock. For a concentration value of 0.05 mg/mL the monolith capacity was 3.640 mg/mL, whereas at 0.20 mg/mL the capacity increased to 6.359 mg sc pDNA/mL gel. These results are satisfactory when compared with other values described for the pDNA binding capacity (≈8 g/L [21]) obtained with a non-grafted dimethylaminoethyl (DEAE) anion-exchange methacrylate monolith. These intermediate capacity values are associated to the fact of being used non-grafted monoliths. Nevertheless, a comparative study between non-grafted and grafted DEAE weak anion-exchange methacrylate monoliths revealed an improvement on capacity, around 17 mg/mL, when it was used the grafted monolith [17]. These outcomes suggest that the CDI monolith capacity could be increased after grafting this monolith. On the other hand, the capacity obtained for this non-grafted CDI monolith is 6 or 12 times higher than the capacity achieved on histidine agarose-based supports with proteins (1 mg/mL) [30] or pDNA (0.5 mg/mL) [27], respectively. Therefore, the CDI monolith emerges as an interesting support to apply on affinity chromatography that is normally associated with low dynamic binding capacity.

The improvement on monolith capacity with the increase of sc pDNA concentration in the feedstock can be explained by the compaction degree that plasmid molecules suffer, leading to a significant reduction on the molecular apparent size, consequently increasing the surface area on the support for molecules adsorption. The compaction phenomenon can be related with the fact that plasmid molecules in more concentrated solutions suffer a significant compression and have a smaller radius of gyration [28]. On the other hand, it was also described that using high salt concentrations, the intermolecular repulsion between DNA molecules can be reduced, allowing DNA to pack more closely on the surface and leading to higher binding capacity [21]. These results are in accordance with previous plasmid-based works using histidine–agarose chromatography [27] and membrane chromatography or filtration [28,31].

At the end of each breakthrough experiments, the pDNA that remained bound to the monolith was recovered by decreasing the ionic strength of the elution buffer. Besides the target molecule was eluted in a small volume and in a concentrate form, also the contact time with the matrix was short. These conditions become an important criterion to be considered for the choice of the support to apply on pDNA purification, given that they avoid the pDNA degradation [14].

In order to quantitatively evaluate the column loading and the dissociation constant (K_d) from adsorption isotherm between the sc pDNA and CDI monolith support, frontal analysis chromatography was used, according to what is described in a recent publication [32]. This method assumes a Langmuirian behavior, and the K_d value was quantified with the equation $1/[C(V_R - V_M)] = (K_d/m_L)(1/C) + (1/m_L)$; where m_L is the total number of available binding sites in the column, V_M is the column void volume and V_R is the volume required to elute a continuously applied concentration of pDNA (C) from the column. The data were analyzed by linear regression. Through of equation we can plot $1/[C(V_R - V_M)]$ vs $1/C$ to obtain the ordinate intercept value corresponding to $1/m_L$ and the slope corresponding to K_d/m_L.

Using previous equation and the breakthrough experiments obtained with CDI monolith at different sc plasmid concentrations, the m_L value was found to be $2.50 \pm 0.23 \times 10^{-9}$ mol and K_d $4.81 \pm 0.21 \times 10^{-8}$ M. Previous works also developed a quantitative affinity chromatography technique for determination of the binding constants characterizing the biospecific adsorption and desorption phenomena [33]. In fact, the risks of irreversible biomolecules adsorption and denaturation are minimized when the value of K_d is between 10^{-4} and 10^{-8} M. By this way, the dissociation constant value obtained was near to 10^{-8} M, revealing a good affinity interaction between the ligand and pDNA, which indicates that CDI monolith is a good affinity support.

3.3.3. Effect of flow rate

To verify if a reduction in the flow rate utilized can result in an improvement of the binding capacity, some breakthrough experiments with a 0.1 mg/mL solution of sc pDNA were conducted at 26.5 and 53 cm/h (Fig. 4). Through analysis of normalized curves obtained for both flow rates, it is clear in Fig. 4 that to the experiment of lower flow rate (26.5 cm/h) is associated a slightly increase on the support capacity for pDNA binding. This result is in agree-

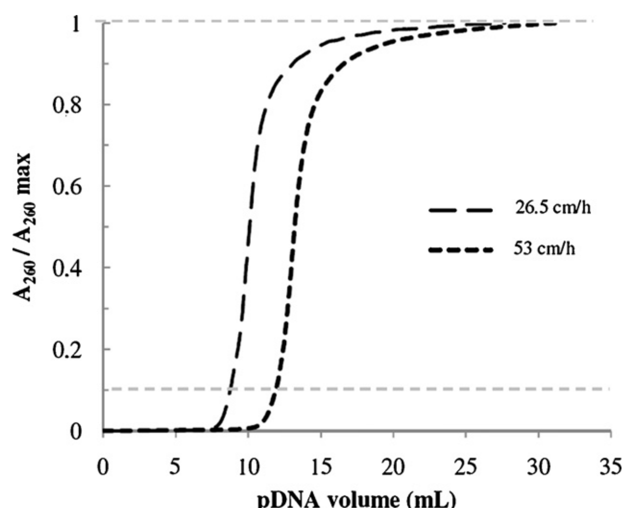

Fig. 4. Breakthrough curves of sc plasmid DNA in CDI monolithic support at different flow rates (26.5 and 53 cm/h). Feedstock: sc plasmid solution, at 0.1 mg/mL, was prepared in 2.5 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer pH 8.0.

ment with a recent publication [27] since the effect of lower flow rate on the retention behavior of pDNA can be related with the increasing contact time between the matrix and the solutes, favoring the attainment of equilibrium and inducing a more efficient binding. Haber and collaborators also found out that sc pDNA becomes less extended at lower flow rates, thus occupying a smaller area and increasing the binding capacity [31].

Curiously, there are some disagreements in the literature about the effect on the flow rate. For example, Bencina et al. [21] demonstrated that flow velocity up to 700 cm/h has no effect on plasmid DBC. Also Krajnc et al. [34] recently reported flow unaffected capacity for a 39.4 kbp plasmid at linear velocity up to 300 cm/h. Otherwise, further extension of this range up to 1000 cm/h performed by Urthaler et al. [14] and Zochling et al. [35] indicated a small decrease of binding capacity, as well as happens in our study but only with a linear velocity of 53 cm/h.

4. Conclusions

The successful implementation of new pDNA-based therapeutic strategies is partially affected by the vector manufacturing process. Due to the singularity of plasmid molecules, its efficient purification is still challenging. The novel non-grafted monolithic support, employed in the present study for the pDNA purification, offers several potential advantages over traditional supports, including higher selectivity and productivity and good capacity. The separation process of plasmid isoforms through a CDI monolith has shown to be flow-independent, involving a specific recognition of the sc isoform. A maximum capacity was obtained when increased concentrations of sc plasmid conformation and decreased revealed linear velocities were used. The obtained K_d value, $4.81 \pm 0.21 \times 10^{-8}$, confirmed that CDI monolith support develops a good affinity interaction with pDNA, showing satisfactory affinity properties as a chromatographic support. Therefore, this glycidyl methacrylate monolith represents a robust purification matrix which enables efficient purification of sc isoform with a high purity degree. This fact opens new possibilities for further application of this chromatographic technology to efficiently isolate sc isoform from other pDNA isoforms and *E. coli* host impurities present in the clarified lysate.

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