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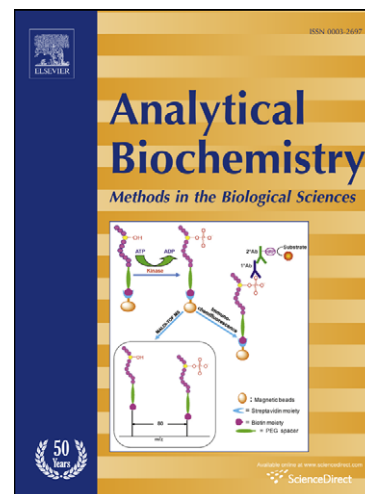
PII: S0003-2697(11)00060-1
DOI: [10.1016/j.ab.2011.01.033](https://doi.org/10.1016/j.ab.2011.01.033)
Reference: YABIO 10322

To appear in: *Analytical Biochemistry*

Received Date: 3 September 2010
Revised Date: 29 December 2010
Accepted Date: 25 January 2011

Please cite this article as: C. Caramelo-Nunes, T. Tente, P. Almeida, J.C. Marcos, C.T. Tomaz, Specific berenil–DNA interactions: an approach for separation of plasmid isoforms by pseudo–affinity chromatography, *Analytical Biochemistry* (2011), doi: [10.1016/j.ab.2011.01.033](https://doi.org/10.1016/j.ab.2011.01.033)

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**Specific berenil–DNA interactions: an approach for separation of plasmid isoforms by
pseudo–affinity chromatography**

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Short title: Berenil as ligand for plasmid isoforms separation

Subject category: Fluorimetric titration / Pseudo-affinity chromatography

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ABSTRACT

Small molecules, like some antibiotics and anticancer agents that bind DNA with high specificity can represent a relevant alternative as ligands in affinity processes for plasmid DNA (pDNA) purification. In the present study, pDNA binding affinities of berberine, berenil, kanamycin and neomycin were evaluated by a competitive displacement assay with ethidium bromide using a fluorimetric titration technique. The binding between pDNA and ethidium bromide was tested in different buffer conditions varying the type and the salt concentration, and was performed both in absence and in presence of the studied compounds. The results showed that the minor groove binder berenil has the higher pDNA binding constant. Chromatographic experiments using a derivatized column with berenil as ligand, showed a total retention of pDNA using 1.3 M ammonium sulphate in eluent buffer. A selective separation of supercoiled and open circular isoforms was achieved by further decreasing salt concentration to 0.6 M and then to 0 M. These results suggest a promising application of berenil as ligand for specific purification of pDNA supercoiled isoform by pseudo-affinity chromatography.

KEYWORDS: small DNA ligands; berenil; minor groove binder; intercalator; pseudo-affinity chromatography

INTRODUCTION

Molecular therapies such as gene therapy and DNA vaccines are increasingly being established as promising and potent alternatives to classical treatments [1]. Both methodologies are based on vector-mediated introduction of a therapeutic nucleic acid molecule in patient selected cells. Although a great amount of work has been reported using viral vectors its safety is a major concern in human studies [2]. Non-viral vectors, like plasmid DNA, are emerging as a better alternative both in terms of safety and easy of production [3]. The expected wide application of these methodologies requires the large-scale production and purification of plasmids. Classical protocols for purification of plasmids used in molecular biology are widely available [4], however and besides the fact that they are not suitable to be applied in large-scale manufacturing, they frequently use toxic reagents that prevent their utilization for therapeutic products purification [5].

Recent years have witnessed an increasing research effort in the development of new methods for plasmid purification. Most of these are based on chromatography processes [6], but other procedures using precipitation [7] and aqueous two-phase systems [8] had also been described. Although affinity methods seem to be very adequate for purification purposes, they had not been extensively explored. Successful cases report the use of proteins as ligands [9], however besides the cost of the ligands, the target plasmid had to bear a sequence with high affinity for these molecules, which limits and complicates the application of these methodologies. Therefore, the use of other ligands, cheaper and sequence independent are required. It was reported the utilization of affinity chromatography (AC) using arginine [10], histidine [11] and lysine [12] as ligands for separation of pDNA isoforms and for selective purification of supercoiled (sc) pDNA from clarified cell lysates [11]. Although amino acid based AC is a promising approach for DNA purification, some drawbacks have to be overcome such as the low capacity of available supports and low recovery yields [13].

Several molecules, like certain antibiotics and anticancer agents, bind DNA with high specificity [14]. Due to the complex structure of double-helical DNA, different binding modes are possible. Besides covalent binding, there are several classes of specific or unspecific noncovalent binding modes, like minor groove binding (MGB), intercalation between base pairs, bisintercalation, major groove binding and a combination of the above [15]. Since the first two are specific for DNA but sequence independent, molecules that bind this way should be very promising for developing affinity purification methods [16]. Examples of these DNA binding molecules include berberine, berenil, kanamycin and neomycin.

Neomycin and kanamycin belong to the family of anti-neoplastic drugs, produced by *Streptomyces* genera [17, 18]. Neomycin binds to guanine bases in the deep major groove of the double helix by an intercalative manner [19]. On the other hand, berenil is a member of the aromatic diamidine class of DNA binding agents, which reversibly binds preferentially to DNA minor groove at central AATT sequence [20]. Finally, berberine is a major isoquinoline alkaloid present in a number of clinically medical plants [21] that binds to the double helix with high affinity, nevertheless the binding mode is not completely clear. The most probable way of binding is a partial intercalation, where a portion of the ligand molecule intercalates into the double helix and the other portion protrudes into the minor groove at the AT-rich sequences [22, 23].

More than a new and unexplored approach, the application of small DNA binders as ligands for pDNA affinity purification seems to be able to improve and simplify currently available protocols and could, in the near future, be implemented in the large scale purification of plasmids vectors for molecular therapies.

In this work, we reported the evaluation of DNA binding affinities of berberine, berenil, kanamycin and neomycin using a model plasmid (pVAX1-LacZ[®]). The binding affinities were determined by a competitive displacement assay with ethidium bromide (EtBr) using a fluorimetric titration technique. The most promising ligand was then used in sc pDNA isoform purification studies by a pseudo-affinity type chromatography.

Materials and methods

Reagents and stock solutions

Ethidium bromide was obtained from AMRESCO (Cochran, OH, USA). Kanamycin was from Calbiochem (San Diego, CA, USA). Berenil, neomycin, berberine and 1,4-butanediol diglycidyl ether were purchased from Sigma-Aldrich (St. Louis, MO, USA). All salts used were of analytical grade.

Sodium chloride and ammonium sulphate solutions were prepared in different concentration in buffer Tris-HCl 0.05 M, pH 7.5 for the fluorimetric measures and in Tris-HCl 10 mM, pH 8 for the chromatographic experiments. A stock solution of ethidium bromide (solution A) in deionised water was prepared with an approximate concentration of 3.0×10^{-4} M. The exact concentration of this solution was determined by diluting a small portion 100 fold and measuring the absorbance at 480 nm ($\epsilon = 5600 \text{ M}^{-1} \text{ cm}^{-1}$). Working solutions were obtained by appropriate dilution with Tris-HCl buffer. The solution B was prepared by diluting solution A with buffer to a concentration of 3.0×10^{-6} M.

Plasmid production and purification

Escherichia coli DH5 α strain harbouring 6.05 Kpb plasmid pVax1-LacZ (Invitrogene, Carlsband, CA, USA) was cultured overnight in Luria Bertani medium supplemented with 30 $\mu\text{g}/\text{mL}$ of kanamycin at 37°C. Cell growth was carried out at same temperature in an erlenmeyer with Terrific Broth medium supplemented with 30 $\mu\text{g}/\text{mL}$ kanamycin. The growth was suspended at late log phase ($\text{OD}_{600} \approx 10$). Cells were recovered by centrifugation and stored at -20°C. Plasmid DNA was purified using the Qiagen plasmid midi kit (Hilden, Germany) according to the manufacturer's instructions. Plasmid quantification was made by measuring the absorbance at 260 nm.

Determination of Fluorimeter Settings

Fluorescence measurements were carried out on a FluoroMax[®]-4 Horiba spectrofluorometer (Edison, NJ, USA), using a cell of 1 cm path length. A cuvette was filled with 2.2 mL of buffer (Tris-HCl 0.05 M + 0.5 M NaCl, pH 7.5), 0.10 mL of plasmid solution (1.5×10^{-3} M in buffer), and 50 μL of solution A and mixed well. The cuvette was then placed in the fluorimeter and the excitation wavelength was set to 525 nm, the excitation slit to 5 nm, and the emission slit to 3 nm. The emission wavelength was set to the value that gave the maximum intensity (599 nm).

Determination of the EtBr binding parameters to DNA

The proportionality constant for free EtBr (k_f), the proportionality constant for EtBr bound to the DNA (k_b) and the binding constant of DNA-EtBr (K) were determined experimentally according to the method described by Strothkamp and Strothkamp [24].

Determination of EtBr binding to DNA in the presence of the studied compounds

The binding constant of EtBr to pDNA in the presence of the four different ligands (K_{obs}) was determined according to the method described by Strothkamp and Strothkamp [24]. A starting aliquot of 75 μ L of ligand solution (neomycin and kanamycin 100 mM in buffer; berberine and berenil 1.0 mM in deionised water) was added the plasmid solution and the fluorescence intensity was measured. This solution was titrated with aliquots of solution A. The titration was repeated with 150 μ L, 225 μ L, 300 μ L, 375 μ L aliquots of ligand solution. The method for determination of k_f , k_b and K_{obs} was repeated for seven different concentrations (0.5 to 2.0 M) of sodium chloride and ammonium sulphate in buffer. The assays were repeated three times and the median of each group of constants was determined for each ligand.

Analysis of Scatchard plots and binding modes

The analysis of the Scatchard plots was made according to Strothkamp and Strothkamp [24]. The influence of the four compounds on pDNA/EtBr binding was determined by measuring the reduction of fluorescence intensity. The amount of EtBr bound per unit of DNA, was determined using a simple binding model. The data was fitted to the Scatchard equation $r/C_f = nK - rK$, where r is the ratio of bound EtBr to DNA base pair concentration, C_f is the concentration of free EtBr, n is the maximum value of r and K is the intrinsic binding constant of the EtBr [24]. The change of fluorescence was calculated considering the ligands as competitive inhibitors. Adding a competitive ligand to a Scatchard analysis produces the relationship $1/K_{obs} = (K'/K)C'_f + 1/K$, where K_{obs} is the slope of the straight line r/C_f according to r , for each concentration of ligand, K' is the binding constant of the ligand to DNA and C'_f is the concentration of free ligand. By plotting the values of $1/K_{obs}$ against concentration of ligand, it is possible to fit a straight line, giving a line with slope K'/K from which one can calculate the value of K' [24].

Preparation of Berenil-Sepharose support

Sepharose CL-6B (Amersham Biosciences, Uppsala, Sweden) was epoxy-activated according to the method described by Sundberg and Porath [25], and coupled to berenil. Initially, Sepharose CL-6B was washed with large volumes of deionised water. To 5 g of moist gel was added 5 mL of NaOH 0.6 M solution, containing 50 mg of NaBH₄, and 5 mL of 1,4-butanediol diglycidyl ether. The slurry was swirled at 25°C for 8 hours using a bath with orbital agitation. The epoxy-activated gel was then washed in a sintered glass funnel with large volumes of deionised water. The gel was then suction-filtered to near dryness and 2 g were added to 4 mL of a 2.0 M sodium carbonate solution containing 500 mg of berenil. After swirling for 16 hours at 70°C, the derivatized Sepharose was washed with large volumes of deionised water and 70% ethanol solution to remove the excess of ligand and sodium carbonate. The derivatized gel was stored at 4°C in deionised water.

Chromatographic Method

Chromatography studies were performed in a Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences, Uppsala, Sweden) at room temperature. A 3 mL column was packed with the berenil-derivatized support and initially tested with different mobile phases, namely 10 mM Tris-HCl buffer, pH 8, with 0.1-3.0 M sodium chloride or 0.5-3.0 M ammonium sulphate concentrations. Prior to the sample application, the column was equilibrated with 1.3 M ammonium sulphate in 10 mM Tris buffer (pH 8.0) at a flow rate of 1 mL/min. Plasmid samples (25 µL) of 900 µg/mL in equilibration buffer were then injected at the same flow rate. The elution of bonded pDNA was carried out by decreasing the ionic strength to 0M (10 mM Tris buffer, pH 8.0). To promote the selective elution of bonded species, first the ionic strength was decreased to 0.6 M ammonium sulphate in 10 mM Tris buffer (pH 8.0) and afterwards to 0 M. The absorbance was monitored at 280 nm. Fractions were pooled according to chromatograms obtained and were concentrated and desalted using Vivaspin concentrators (Vivascience) and analyzed by gel electrophoresis (100V for 40 min.) using 1% agarose gel in TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA, pH 8.0) in the presence of 0.5 µg/mL ethidium bromide. After the chromatographic runs, the column was washed with at least 5 bed volumes of deionised water.

Results

Analysis of pDNA-ligand binding affinity

The binding of berberine, berenil, kanamycin and neomycin to DNA plasmid pVAX1LacZ was studied by a fluorimetric titration technique. The influence of sodium chloride in this interaction was evaluated using seven different concentrations of salt (0.5 M to 2.0 M) in 0.05 M Tris, pH 7.5. The highest affinity was found for 1.0 M of sodium chloride, being neomycin the only exception, for which 0.5 M gave the highest affinity constant (67.36 M^{-1}). Therefore, the best binders at the concentration of 1.0 M of sodium chloride were berenil (12849 M^{-1}) and berberine ($7110,86 \text{ M}^{-1}$) with affinity constants about two orders of magnitude higher than kanamycin ($33,25 \text{ M}^{-1}$) and neomycin ($55,73 \text{ M}^{-1}$).

Overall, the relative order of affinity was berenil > berberine > neomycin > kanamycin. Berenil presents a wide variation of the affinity constant with salt concentration, with values ranging from 2223 M^{-1} to 12849 M^{-1} (Fig. 1A) For the other compounds tested the variation observed was much slighter (data not shown).

The affinity of berenil to pDNA was also tested in the presence of ammonium sulphate, which enhances hydrophobic interactions. The variation of K' with this salt (Fig. 1B) showed a different behaviour and magnitude with values ranging from 3833 M^{-1} to 5453 M^{-1} . The maximum value was found with a salt concentration of 2.0 M but was much smaller than the one obtained with sodium chloride (12849 M^{-1}). Moreover, there was a broad amplitude of K' values obtained with sodium chloride compared to ammonium sulphate where K' showed small variations with salt concentration (Fig. 1).

Chromatographic Studies

Chromatographic studies were performed to test berenil as an affinity ligand, since this compound showed the highest pDNA binding constant. Moreover, berenil structure makes it a natural candidate to be directly bonded onto an activated support, unlike berberine which has to be submitted to structure modifications in order to introduce suitable functional groups to make the immobilization possible. Thus, the derivatized support for the chromatographic studies was prepared by covalent coupling of berenil on Sepharose CL-6B, previously activated using 1,4-butanediol diglycidyl ether to introduce epoxy groups in the hydroxylic polymer agarose (Fig. 2). Nevertheless, the epoxidation of berenil established through the amine moiety of amidine, the imine linkage or even by both cannot be disregarded.

Initially, the retention profile of pDNA on berenil-Sepharose CL-6B column was studied under the influence of different sodium chloride concentrations (0.1 to 3.0 M) in eluent buffer, considering the results obtained with the fluorescent measurements. However, the pDNA was not retained on the column in these conditions. On the other hand, the experiments performed with ammonium sulphate showed that the maximum retention of pDNA in the column was obtained with 1.3 M of salt and the elution was performed simply by decreasing the salt concentration in eluent buffer to zero. In order to determine the specific binding conditions of supercoiled (sc) and open circular (oc) isoforms, several binding/elution experiments were performed. The data showed that the epoxy-berenil support interacts differently with the plasmid isoforms. In fact, oc plasmid was eluted from the column with the decrease of the ionic strength of the buffer to 0.6 M of ammonium sulphate, whereas the sc isoform remains bound, eluting only when the salt concentration in eluent was decreased to zero (Fig. 3).

The analysis of the elution fractions by agarose gel electrophoresis (Fig. 3) confirms that peak 2 eluted with 0.6 M ammonium sulphate corresponds to the oc isoform (lane 2), whereas peak 3 was attributed to the sc isoform (lane 3). Control experiments using a non-derivatized column, submitted to the same coupling preparation process but in absence of berenil, did not result in any measurable retention of pDNA on the column even using a different salt concentration in the eluent.

Discussion

Differences between interaction types

The magnitude of the binding constants (K') varies widely for the different compounds. All showed a competitive behaviour in relation to EtBr, especially berenil and berberine, which have the highest K' for pDNA. Considering the binding mode of each of the four compounds, results showed that while for the MGB (berenil) the pDNA association constant was in the range of 10^4 M, for intercalators (neomycin and kanamycin) was in the range of 10^1 M. The partial intercalator (berberine) showed an association constant value in the range of 10^3 M. The results suggests that the MGB is the most promising compound for use as ligand in sc isoform purification from the other pDNA isoforms, what could be rationalized thinking that in sc the access of the ligands should be easier to the minor groove than to the space between the base pairs.

Groove binding differ from intercalation at least in one significant way. Structural evidence suggests that there is little change in the DNA conformation upon binding of a groove binding agent. In general, groove binding consist of only two steps: the first would correspond to the transfer of the ligand from solution to the DNA minor groove, secondly, the ligand can form noncovalent molecular interactions with DNA groups accessible in the groove, that influence the excellent structural fit of these ligands [26,27]. In contrast, intercalators bind DNA by unwinding the helix and unstacking the base pairs [15], consuming energy to form the intercalation cavity [28], which can therefore justify the lower binding constants of these compounds.

Nature of pDNA-free ligand interaction

Both partial intercalator and MGB tested in this study feature aromatic ring systems that are hydrophobic in nature. Transfer of these rings from aqueous solution into the interior of the minor groove of DNA should be energetically favourable [26]. So, it is expected that the binding of these ligands with pDNA would have a large hydrophobic contribution, but those may not be the only interactions involved in the binding. The results indicate that the binding of intercalators or MGB to DNA is salt dependent, but only at high salt concentrations (1M or higher for both salts) it is possible to observe strong binding affinities. It seems that the predominant forces involved in pDNA-ligand binding are hydrophobic

interactions [29], however the electrostatic contribution due to a salt effect alone cannot be neglected. It is also important to consider the type of salt used to study the interaction, since a salt as sodium chloride in low concentrations increases the electrostatic interactions, while ammonium sulphate in high concentrations enhances the hydrophobic interactions.

A more comprehensive study was dedicated to the binding of berenil to DNA, since this compound showed the highest K' . First it is important to establish the interactions involved in berenil binding to DNA. The DNA binding affinity of this ligand has been attributed to several factors like electrostatic interactions with the negative electrostatic potential specially associated with AT sequences [30], hydrophobic contacts between the phenyl rings and the hydrophobic regions of the backbone and the triazene group neighbouring the polar phosphodiester groups [31]. The amidines form hydrogen bonds with thymine and/or adenine acceptor groups of the bases at the floor of the groove [32].

At working pH (7.5 and 8.0) both amidine groups of berenil are protonated ($pK_a = 11$), providing electrostatic contributions to the complex energetics through phosphate interactions [33]. The relative importance of these interactions in determining the binding of berenil cations to DNA is reflected in the salt dependence of the constant [34], but only with a salt that enhances the electrostatic interactions. Our results showed that berenil K' changed considerably with the variation of sodium chloride concentration, while this constant is not greatly affected by ammonium sulphate concentration (Fig. 1). So at least one of the putative binding modes of berenil to pDNA depends more on the charged ends, which can be explained by the presence of strong electrostatic interactions [35] and high constant values.

The nonpolyelectrolyte contribution to berenil-pDNA binding should not change appreciably with the ionic strength. This means that hydrogen bonding and van der Waals interactions will not vary significantly [35]. On the other hand, with ammonium sulphate in the eluent, the binding of berenil to pDNA depends more on the hydrophobic than electrostatic interactions.

Interaction between pDNA and berenil bonded to the support

After the fluorimetric titration measurements, a higher retention of pDNA using sodium chloride over ammonium sulphate could be expected. Additionally, the variation of binding constant values, with different concentration of sodium chloride, suggested a simple method for binding and elution of the plasmid from derivatized support in pseudo-affinity chromatography by a simple decrease of buffer salt concentration. Instead, the experimental studies showed that retention of the pDNA was achieved only using a relatively high ammonium sulphate in the buffer solution. The high salt concentration increases

the binding of biopolymers to the stationary phase by hydrophobic interactions, which are attenuated upon reducing the ionic strength of the eluent, in the order of decreasing the hydrophobic character [36].

Many causes may be behind this peculiar behaviour of berenil after immobilization onto the matrix. It is expected that berenil binds to epoxi groups through mainly one of the amidine groups, the same that are responsible for hydrogen bonds and electrostatic interactions with DNA bases [32]. With sodium chloride, the strongest forces involved in the binding are the electrostatic interactions dependent on both charged ends of berenil [34, 35]. So, the fact that one of those ends is involved in the binding to epoxi groups of the support is clearly going to negatively affect the binding of berenil to pDNA. Reducing or removing the charge of the molecule, results in a systematic reduction of the overall binding, because the electrostatic contribution to the total interaction energy is expected to be lowered [30]. Besides this fact, the contribution of the hydrogen bonds to the binding [32] is different for both ends of the molecule. At one end of the ligand, the amidine group establishes direct hydrogen-bonded contact with the DNA base, however the other amidine group does not make direct interactions with DNA, instead a water molecule mediates between them [31]. This water-mediated contact is likely to be weaker than the direct one [37] so, it is possible that berenil can not establish the binding only with one end performing the hydrogen-bonded contact, in part because of the stereochemical hindrance, but mainly because that end may not establish direct and strong contacts with the groove. Therefore, the reasons for the lack of retention of pDNA on the derivatized column using sodium chloride may include: i) decrease of the electrostatic energy contribution to the binding by decreasing the charge of the molecule and ii) impediment of establishing enough and/or direct and therefore more efficient hydrogen bonds.

With ammonium sulphate, the electrostatic interactions and hydrogen bonds are not as crucial to the binding as the hydrophobic interactions [31]. So, apparently the fact that one amidine group was bonded to the epoxi groups of the support was not an impediment for the binding of berenil to pDNA. It appears that the region of berenil molecule that establishes hydrophobic contacts with pDNA remained able to interact with the nucleic acid molecule. These studies let us to conclude that the apparently weaker hydrophobic interactions are enough to promote the binding of pDNA to immobilized berenil.

In addition, it was observed a difference in retention behaviour of the two pDNA isoforms. Since phosphate and sugar groups are equally exposed in both isoforms, the observed selectivity must be due to stronger interactions of berenil with the bases of the groove of sc pDNA, but not with the bases of oc isoform. In fact, as a consequence of deformations induced by torsional strain, the bases of the sc isoform are more exposed than the ones of the oc isoform [38]. Moreover, the DNA structure is sensitive to the

composition and concentration of the ion atmosphere. In solution, salt cations surrounding the double helix can neutralize the anionic charges of the sc DNA molecule so that the repulsions of the chains will decrease. When the salt concentration is higher, the repulsion is smaller [39]. Thus, at high salt concentrations the sc DNA molecule adopts a highly compact and bent interwound state [40], promoting an overexposure of the bases. At low salt, the DNA supercoils are much more open and loosely interwound [40], weakening the binding between the ligand and the less exposed bases, promoting the elution of the sc isoform.

The control experiments unequivocally identify berenil bonded onto the activated Sepharose as the ligand responsible for the retention and separation of the two pDNA isoforms. Moreover, the retention and separation of pDNA isoforms was achieved using a lower salt concentration comparing to other pseudo-affinity ligands [38], thus avoiding the drawbacks of the use of high salt concentration in chromatographic processes [13].

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Conclusions

In the present study, four small DNA ligands (berenil, berberine, neomycin and kanamycin) were analysed for their binding affinity to pDNA using a fluorimetric titration technique. Among the ligands evaluated in a free form, berenil was found to have the highest affinity constant for pDNA. A different binding mode may be the reason for the stronger binding affinity of berenil compared to the other ligands, since it is a MGB in opposition to the other three intercalators. The essential mechanism seems to involve not only hydrophobic interactions between the ligand and the pDNA molecules but also other nonpolar and polyelectrolyte contributions important to the binding.

The influence of the type and salt concentration on the ligands interaction with pDNA demonstrated that the binding affinity is influenced by both parameters. The results demonstrated that the interactions between berenil and pDNA in presence of ammonium sulphate are enough to promote a total retention of this biopolymer and a selective separation of the oc and sc isoforms. In fact, one of the most remarkable results of the present work is related to the specific recognition of sc isoform by this ligand. As far as we know, this is the first study showing the use of berenil as an efficient alternative to existing ligands for sc plasmid isoform purification. Additionally, the retention and separation of pDNA isoforms was achieved using a lower salt concentration comparing to other pseudo-affinity ligands, thus favouring the environmental sustainability.

The promising results obtained using berenil as ligand indicate that this could be an excellent choice for the future purification of sc pDNA from *E. coli* host impurities present in the clarified lysates, for further application in gene therapy and DNA vaccines.

Acknowledgements

C. Caramelo-Nunes acknowledges a fellowship (SFRH/BD/64918/2009) from Portuguese Foundation for Science and Technology (FCT).

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Figure Legends

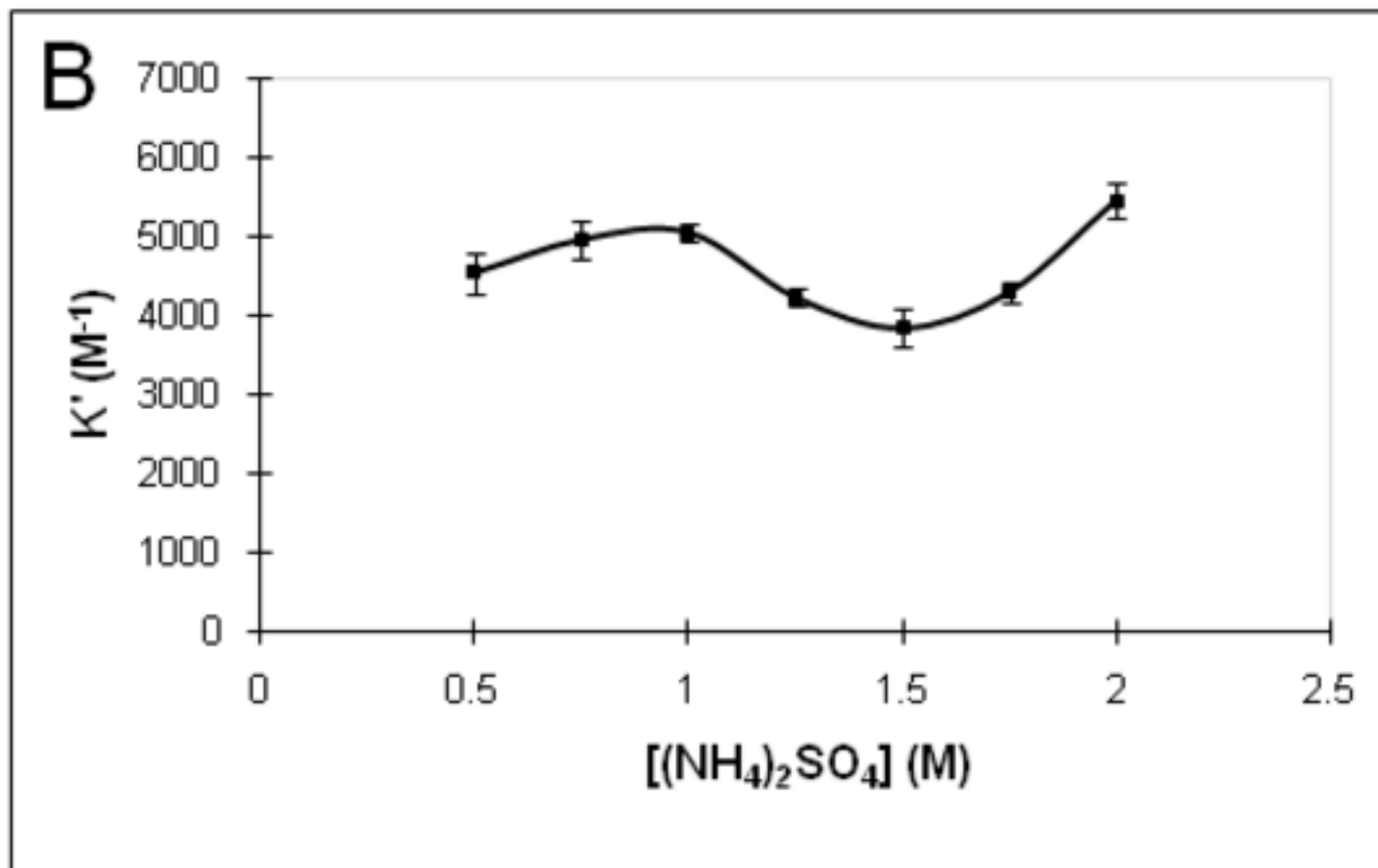
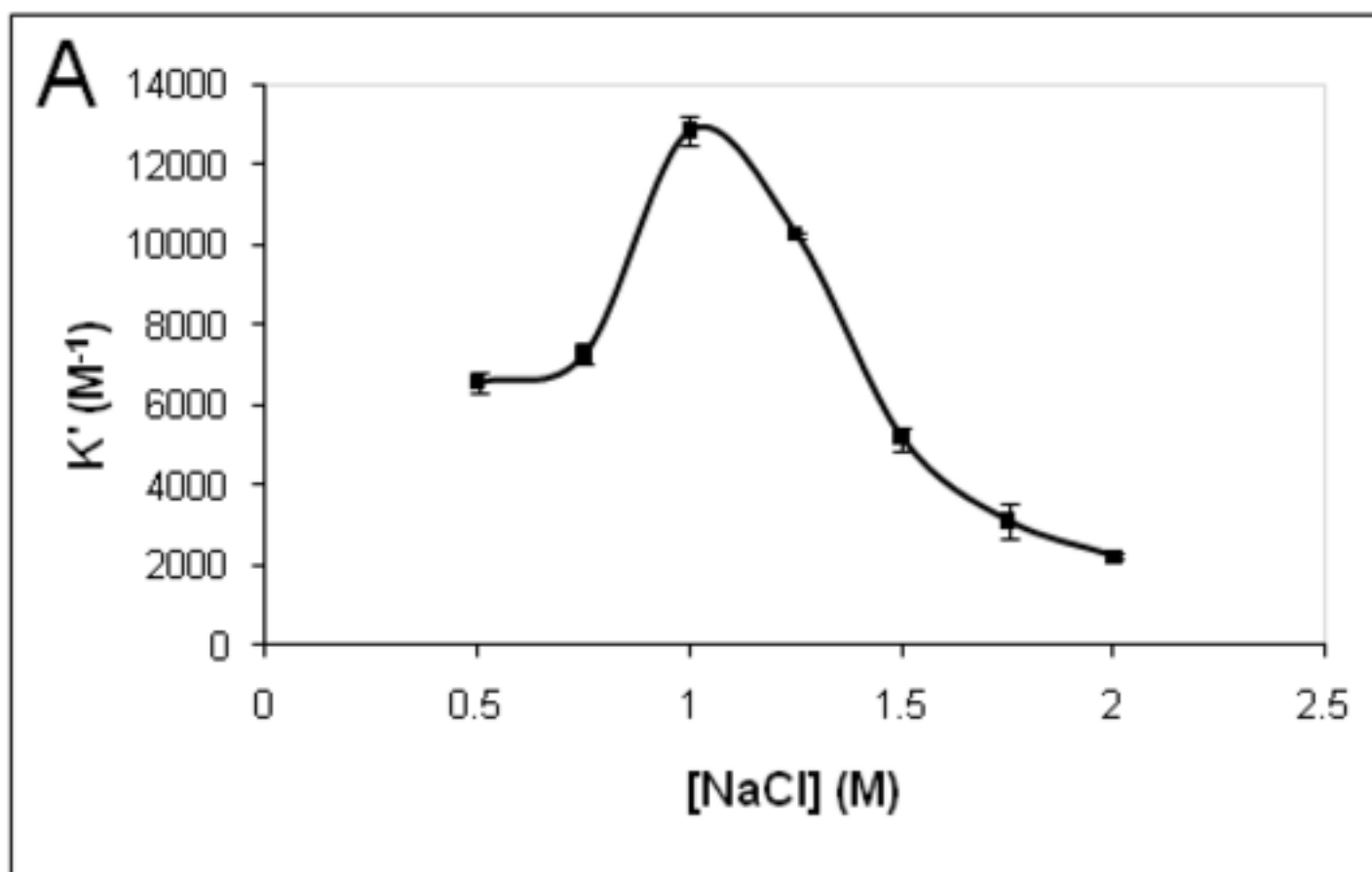
Fig. 1. Variation of the binding constant (K') of berenil with: A – sodium chloride and B - ammonium sulphate concentration.

Results show mean \pm standard deviations ($n = 3$).

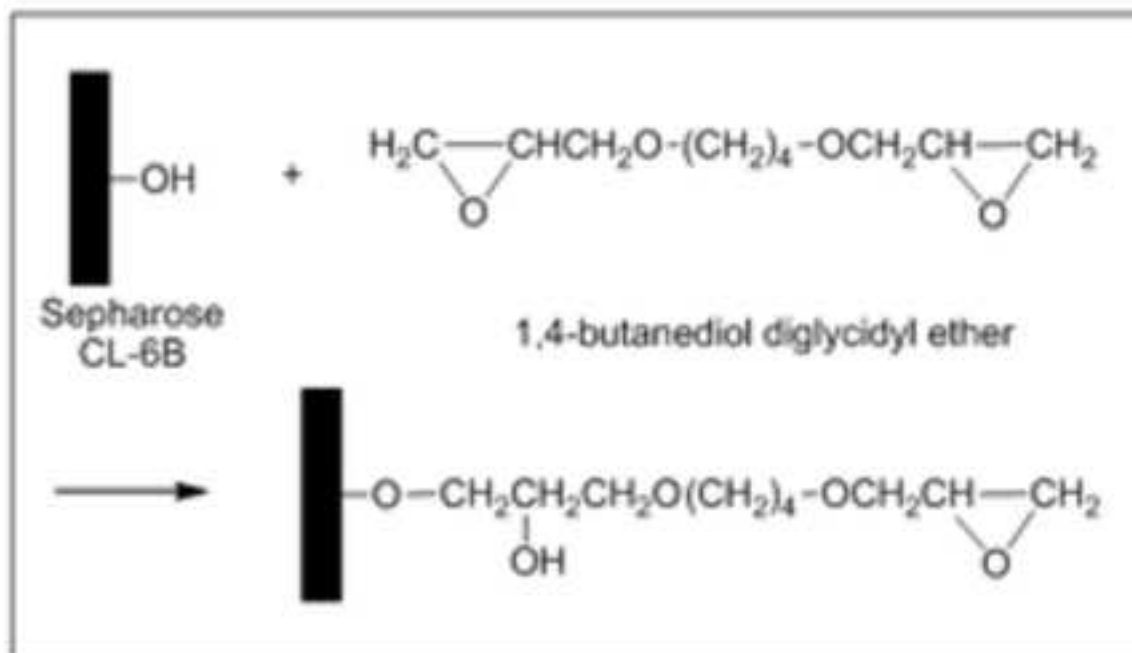
Fig. 2. Epoxi activation of Sepharose CL-6B (A) and derivatization with berenil (B).

Fig. 3. Chromatographic separation of pDNA (25 μ L, 900 μ g/ml) isoforms on berenil derivatized column and agarose gel electrophoresis analysis of the eluted fractions. Lane M: molecular weight markers; lane A: pDNA sample injected onto the column (oc + sc); lane 1 – Fraction collected after injection with 1.3M $(\text{NH}_4)_2\text{SO}_4$; lane 2: sample collected from the first peak (oc) eluted with 0.6M $(\text{NH}_4)_2\text{SO}_4$ and lane 3: sample collected from the second peak (sc) eluted with 0M $(\text{NH}_4)_2\text{SO}_4$.

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A



B

