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Purification of plasmids by triplex affinity interaction

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ABSTRACT

Production of pharmaceutical grade plasmid DNA is an important issue in gene therapy. We developed a method for affinity purification of plasmids by triple helix interaction. This method is based on sequence-specific binding of an oligonucleotide immobilized on a large pore chromatography support to a target sequence on the plasmid. Using design criteria derived from thermodynamic data, we produced a 15mer target sequence which binds strongly to the affinity support under mildly acidic conditions. Plasmid DNA was purified from clarified *Escherichia coli* lysate by incubation with the affinity beads at pH 5.0 and high NaCl concentration. After extensive washing of the beads, purified plasmid DNA was eluted with alkaline buffer. The purified plasmid showed no RNA or cell DNA contamination in HPLC analysis and total protein concentration was reduced considerably. Due to its mechanical stability and porosity this support can be used in a continuous affinity purification process, which has a high potential for scale up.

INTRODUCTION

Production of large amounts of pure plasmid DNA from bacterial cultures has become an important area of interest. While plasmid preparations have traditionally been used for many applications in cloning and molecular genetic research, the use of 'naked' or lipid-coated plasmid DNA for non-viral gene therapy (1–5) as well as for DNA vaccination (6) has increased the requirements for such preparations with regard to both quality and quantity. Plasmid purification is essentially aimed at eliminating cellular components of the host organism, such as bacterial proteins, lipids, lipopolysaccharides and nucleic acids, while avoiding the introduction of contaminants. Chromosomal DNA fragments and RNA are especially difficult to separate from plasmids due to the similarity in their physical and chemical structure.

Laboratory scale purification methods have generally relied on the use of toxic chemicals (EtBr, phenol, CsCl) or animal-derived enzymes (RNase), all of which might raise concerns about residues in the final product. Therefore, various new processes have been developed which rely on chromatographic unit operations such as gel filtration (7) or ion exchange (8–10). In view of the unmatched specificity of affinity purification methods, we propose the use of triplex affinity interaction for downstream processing of plasmid DNA.

Triple helix formation has been known for several decades and triplex structures have been very well characterized (for reviews see 11,12). The best characterized triplex is formed between a double-stranded homopurine-homopyrimidine helix and a single-stranded homopyrimidine strand. In this type of triple helix, the third homopyrimidine strand binds to the major groove, parallel to the homopurine strand of the Watson–Crick double helix, via Hoogsteen hydrogen bonds. The third strand thymine (T) recognizes adenine-thymine (A·T) base pairs forming T·A·T triplets and the protonated third strand cytosine (C^+) recognizes guanine-cytosine (G·C) base pairs forming C·G·C^+ triplets.

Several authors characterized the thermodynamics of triplex formation for different sequences and pH values (13,14) and the influence of pH, buffer species and salt concentration on triplex thermodynamics (15,16). These studies found that for neutral to slightly acidic buffer conditions triplex formation is enthalpically driven and H bond formation is the major driving force.

The fact that the cytosines in the third strand have to be protonated leads to a decrease in melting temperature T_m with increasing pH (13,14,16,17). Frequency and distribution of C·G·C^+ triplets in the target sequence therefore have a strong influence on pH dependence and stability of the triplex. The less C·G·C^+ triplets there are in a given sequence the higher the binding constant is at mildly acidic to neutral pH. On the other hand, it was found that triplex formation occurs at pH values above the pK_a of free cytosines (pK_a 4.8) (14,16,17) and that C·G·C^+ triplets increase the stability of pure T·A·T triplex structures significantly (17).

Melting experiments at different salt concentrations further showed that T_m increases with salt concentration (16,17), which can be attributed to cations shielding the coulombic repulsion between the sugar–phosphate backbones.

These findings strongly influence specificity and stringency of triplex formation and, therefore, the design of possible target sequences. Specificity in this context means a large destabilization upon binding to a mismatching DNA sequence (large free energy penalty) (18). Stringency, on the other hand, refers to a set of conditions in which perfect complexes between ligand and ligate are stable and imperfect complexes are not (18). Different authors found free energy penalties for mismatches of from 2.5 to 4.0 kcal/mol, resulting in a decrease in melting temperatures for defect triplexes of 12 to >20°C per mismatch (17,18).

There are few applications of triplex affinity isolation described in the literature. Kumar and Rustogi (19) used an oligonucleotide linked to a solid support to isolate a loop-forming oligonucleotide by triplex formation. Ito *et al*. (20) isolated a linearized plasmid containing a 45 bp T-C repeat with a 5′-biotinylated oligonucleotide

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20mer. Very efficient enrichment was obtained at pH 5.0. Recently, a plasmid purification method based on triplex affinity chromatography was investigated (21) and specific binding of supercoiled plasmid containing the target sequence to a column packed with oligonucleotide affinity beads was found.

In another study (T.Schluep and C.L.Cooney, submitted to *J. Chromatogr. A*) we described the production of a large pore affinity support in which the triplex-forming oligonucleotide ligand is immobilized by its 5′-end to Sephacryl S-1000 SF beads (Pharmacia Biotech, Uppsala, Sweden) via a hydrophilic 16 atom spacer arm. In this paper we report the design and cloning of the target sequence into the plasmid pUC19. We further demonstrate binding of this plasmid to the affinity beads and triplex affinity purification from clarified lysate.

MATERIALS AND METHODS

Luria broth (LB) was from Difco laboratories (Detroit, MI). *Escherichia coli* subcloning efficient DH5α competent cells, plasmid pUC19 and molecular weight marker *Hin*dIII digest of λ phage DNA were from Gibco BRL (Gaithersburg, MD). Restriction endonucleases *Sma*I and *Nco*I were from New England Biolabs (Beverly, MA). All salts were analytical grade.

Affinity support

A detailed report on the chemistry involved in production of the affinity support is given elsewhere (T.Schluep and C.L.Cooney, submitted to *J. Chromatogr. A*). In brief, we synthesized a novel cyanoethyl-protected glycolic acid methylester phosphoramidite. This compound was added in the final cycle of the synthesis of the triplex-forming ligand oligonucleotide, 5′-TCT TCT TTC CTC TTT-3′, on an automated, phosphoramidite-based DNA synthesizer. After treatment with TMA the oligo was cleaved off the column and deprotected by standard procedures. The resulting 5′-carboxylic acid functionalized oligonucleotide was reacted with 3-amino-1,2-propanediol in the presence of carbodiimide (DEC) to yield the glycol-15mer, a stable precursor of the reactive aldehyde-15mer. Sephacryl S-1000 SF beads were functionalized with adipic acid dihydrazide by the cyanotransfer method (22). Glycol-15mer was cleaved oxidatively by sodium periodate to give aldehyde-15mer and coupled to the Sephacryl hydrazide beads by reductive amination in the presence of $NaCNBH_3$. This procedure was optimized to give an affinity support with up to 4.9 nmol immobilized ligand/ml beads.

Plasmid construction

Plasmid pTS2 was constructed by insertion of the polypurine-polypyrimidine target sequence in the multiple cloning site of pUC19 using standard molecular biology protocols (23). The two 5′-phosphorylated oligonucleotide 21mers d(5′-CCA TGG AGA AGA AAG GAG AAA-3′) and d(5′-TTT CTC CTT TCT TCT CCA TGG-3′) were custom manufactured by Gibco BRL and annealed to form a double strand containing a *Nco*I cutting site not present in pUC19. This annealed oligo was inserted blunt endedly into the *Sma*I cloning site of pUC19. *Escherichia coli* DH5α competent cells were transformed with the ligation mix and plated on LB plates containing 100 µg/ml ampicillin. Clones containing the plasmid with the target sequence were identified by restriction analysis with *Nco*I and *Sma*I. The identity and orientation of the insert were confirmed by fluorescent DNA sequencing on an Applied Biosystems (Foster City, CA) Model 377 DNA Sequencer using both the M13 forward and M13 reverse sequencing primers. Sequencing confirmed insertion of the complete target sequence, with the polypyrimidine strand located in the R+ strand of pUC19.

Purified plasmid preparations

An *E.coli* DH5α starter culture containing plasmid pTS2 was grown in 100 ml LB (200 µg/ml ampicillin) overnight at 37°C in a 500 ml shaker flask. An aliquot of 800 ml fresh LB was added to the overnight culture and incubated under the same conditions in two 2 l shaker flasks until the OD_{600} reached 1.5. Lysis and primary plasmid isolation were performed using a modified alkaline lysis procedure as described before (10). Final purification was performed on a Qiagen (Hilden, Germany) Megaprep anion exchange column according to the supplier's recommendations. Plasmid pTS2 was precipitated with isopropanol (0.7 vol) and resuspended in 1 ml TE buffer (10 mM Tris, pH 8, 1 mM EDTA). Plasmid concentration was determined with a Hewlett Packard (Waldbronn, Germany) 8452 diode array spectrophotometer at 260 nm (1 A_{260} = 50 µg DNA/ml) and found to be 737 µg/ml.

For the control plasmid pUC19, an *E.coli* DH5α starter culture was grown in 40 ml LB (100 µg/ml ampicillin) at 37°C for 8 h in a 250 ml shaker flask. An aliquot of 1.5 l fresh LB was added to this starter culture and cultivated under the same conditions in two 2 l and one 4 l shaker flasks until the average OD_{600} reached 1.1. Plasmid pUC19 was then purified using a Qiagen Megaprep kit according to the instructions of the manufacturer. pUC19 was then resuspended in 1 ml TE buffer and the plasmid concentration determined by spectrophotometer at 260 nm to be 805 µg/ml.

Clarified lysate

Escherichia coli DH5α cultures transformed with pTS2 were grown in 100 ml LB (100 µg/ml ampicillin) overnight at 37°C in a 500 ml shaker flask. Cells were harvested by centrifugation at 6000 *g* and 4°C for 15 min and resuspended in 4 ml 50 mM Tris, pH 8, 1 mM EDTA. Cells were lysed by addition of 4 ml 200 mM NaOH, 1% SDS and incubation for 5 min at room temperature. Then 4 ml chilled 3 M potassium acetate, pH 5.5, was added and the lysate was centrifuged for 30 min at 20 000 *g* and 4°C. The supernatant was recentrifuged for 10 min under the same conditions to give 12 ml clarified lysate.

Triplex affinity purification of plasmids

A sample of 0.8 ml triplex affinity beads was equilibrated with and suspended in binding buffer (0.2 M sodium acetate–acetic acid, 2 M sodium chloride) with different pH values between 4.5 and 5.5 to give a total volume of 1.5 ml. The plasmid (35–52 µg, 1 vol) was diluted with 2 vol 0.3 M sodium acetate–acetic acid, 3 M sodium chloride to give the same salt concentration and pH as the binding buffer. For clarified lysate, 1 ml lysate was mixed with 2 ml 0.3 M acetate buffer, 3 M sodium chloride, pH 4.5, to give a pH of 5.0. In one case the pH of the clarified lysate was first adjusted to a value of 4.5 with glacial acetic acid before addition of the high salt buffer. The plasmid was then allowed to bind to the affinity beads for 2 h at room temperature with moderate shaking on a spinning wheel. The bead suspension was then added to a column (7 × 50 mm) and packed under gravity. The beads were washed with 9 ml loading buffer until no material was

seen in UV absorption scans of 1 ml wash fractions. Gravity flow elution was performed by addition of elution buffer (1 M Tris, 0.5 mM EDTA, pH 9) with collection of 0.3 ml fractions. Plasmid content and purity of fractions collected was determined by ion exchange HPLC (Fig. 2B and Table 1) and agarose gel electrophoresis (Fig. 3).

Melting temperature experiments

Plasmid preparations were bound to the affinity beads in loading buffer with a pH of 5.0 for 2 h at room temperature and the column packed and washed with loading buffer as described above. The column was subsequently incubated for 30 min at 30°C by submersion in a water bath. After this period the column was removed from the water bath and quickly washed with 1 ml loading buffer at the same temperature. This procedure was then repeated at 5°C temperature increments up to 70°C. The column was then cooled to room temperature and eluted with elution buffer. Fractions collected were analyzed by anion exchange HPLC.

Agarose gel electrophoresis

Agarose gels were run in a QSH unit from IBI (New Haven, CT) coupled to a Pharmacia (Piscataway, NJ) EPS 200 power supply. Electrophoresis of 1% agarose (Sigma Chemical Co., St Louis, MO) gels was carried out in TAE (40 mM Tris base, 20 mM sodium acetate, 2 mM EDTA, pH 8.3) at 65 V for 1 h. Electrophoresis gels were stained for 1 h with ethidium bromide (0.65 µg/ml), destained for 45 min with water and then visualized and photographed under UV light (Fig. 3).

Total protein analysis

Total protein content of clarified lysates was measured using the Pierce BCA assay (Rockford, IL), which is not sensitive to the high SDS levels present. Total protein concentration in triplex affinity-purified plasmid samples was measured by the Bradford assay (BioRad, Richmond, CA), which has good sensitivity at low protein concentrations.

HPLC analysis of plasmid DNA

HPLC analysis was carried out using a Beckman (Fullerton, CA) System Gold Programmable Solvent Module 126, a System Gold Diode Array Detector Module 168, a Hitachi AS 400 Autosampler and System Gold Software (V 8.10) running on a PC. Anion exchange analysis of plasmids was performed on a column (4.6 × 100 mm) packed with Poros 20 QE (Perseptive Biosystems, Framingham, MA) as described before (10). Mobile phase A was 10 mM Tris, pH 8, 1 mM EDTA and mobile phase B was 10 mM Tris, pH 8, 1 mM EDTA, 2 M NaCl. Salt concentration was 0.5 M for 2 min, followed by a linear gradient from 0.5 to 2 M over 5 min and was finally held at 2 M for 2 min. The flow rate was 2 ml/min and absorbance was monitored at 260 nm (Fig. 2).

RESULTS

Principle of triplex affinity purification

The procedure is schematically illustrated in Figure 1. In the adsorption stage, triplex formation occurs between the target sequence on the plasmid and the immobilized polypyrimidine oligonucleotide at moderately acidic pH (4.5–5.5). In this pH

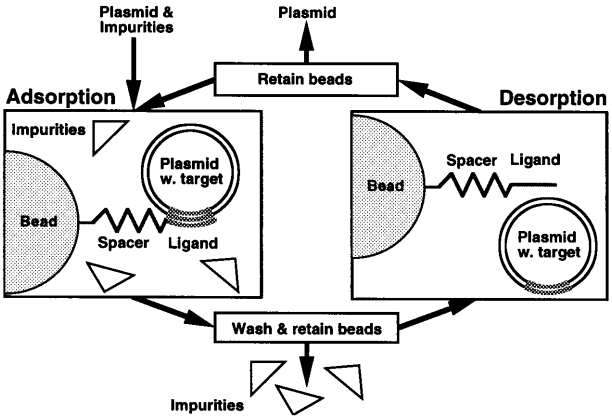

Figure 1. Principle of triplex affinity purification of plasmids. Adsorption and washing is performed under moderately acidic conditions and high salt concentration. Desorption is achieved by shifting to alkaline buffer.

range triplexes are stabilized due to protonation of the N3 position of cytidine (pK_a 4.8) to form C·G·C$^+$ triplets. High sodium chloride is added to the adsorption buffer as it stabilizes triplex formation and reduces non-specific binding of proteins or cell DNA to support, ligate or ligand. Washing is done with large volumes of adsorption buffer. Desorption is achieved by shifting to alkaline pH. Similar conditions have been used by different authors for optimal triplex formation (20,21). This process can be performed in a packed column, as a batch adsorption process, followed by transfer to a column for washing and elution (as here) or by continuous affinity recycle extraction (CARE). CARE was shown to offer a high degree of purification, good recovery yield and a high potential for scale up (24).

Target sequence design

Using thermodynamic data from various authors, as mentioned in the Introduction, we designed a 15mer polypurine target sequence, 5′-AGA AGA AAG GAG AAA-3′, to provide strong triplex formation under the chosen buffer conditions while being selective towards the *E.coli* K12 genome. After adding a unique *Nco*I cutting site to the target sequence for easier detection of correct clones, the plasmid pTS2 was constructed by insertion of the target sequence into pUC19. A homology search on pUC19 showed that it contained a 9mer homologous sequence (5′-AAG GAG AAA-3′) at position 215. This gave us the possibility to explore stringent binding conditions for pTS2 by using pUC19 as a control plasmid.

Characterization of affinity binding

Production of the large pore affinity support is briefly described in Materials and Methods. A detailed report on the immobilization chemistry is published elsewhere (T.Schluep and C.L.Cooney, submitted to *J. Chromatogr. A*). Free hydrazide groups (pK_a 2.9) are present on the affinity support, which might raise concerns about ion exchange properties of the affinity support at acidic pH. Non-specific binding of plasmid DNA to the affinity beads was therefore measured by incubation of hydrazide functionalized beads (2.38 µmol hydrazides/ml beads, no immobilized oligonucleotides) with preparations of purified pUC19, as shown in Table 1. While at pH 4.5 weak non-specific binding of plasmid to the support was seen, no non-specific binding was detected at pH 5.0.

Table 1. Triplex binding experiments with various plasmid preparations

Beads	Plasmid	pH	Plasmid loaded (µg)	Plasmid eluted (µg)	Yield (%)
Hydrazide[a]	pUC19	4.5	51.5	0.6	1.2
Hydrazide	pUC19	5.0	51.5	0.0	0.0
Affinity[b]	pTS2	4.5	36.2	22.4	62.0
Affinity	pUC19	4.5	42.7	16.8	39.4
Affinity	pTS2	5.0	34.7	13.5	38.8
Affinity	pUC19	5.0	52.4	5.4	10.3
Affinity	pTS2	5.5	39.7	9.9	24.8
Affinity	pUC19	5.5	50.6	8.6	16.9
Affinity	Lysate	5.0	4.8[c]	1.8[d]	36.6
Affinity	Lysate	4.5	5.0[c]	1.4[d]	27.8

All plasmid preparations, except the lysates, were previously purified by conventional methods. Conditions were as described in Materials and Methods.
[a]0.85 ml Sephacryl hydrazide beads without oligonucleotide (2.38 µmol hydrazides/ml beads).
[b]0.8 ml Sephacryl affinity beads (4.9 nmol oligo/ml beads).
[c]Amount of plasmid in the lysate was determined by RNase digestion, precipitation with ethanol and HPLC analysis of the resuspended plasmid.
[d]Total supercoiled + denaturated sc plasmid.

Accessibility of pTS2 to the ligands on the large pore affinity support was determined by residence time distribution experiments with a column (1 × 31.4 cm) packed with non-functionalized Sephacryl S-1000 SF. Injections of acetone and clarified lysate preparations of plasmid pUC18, which is the same size as pTS2 (2.7 kb, 1.8 × 10^6 Da), showed that 34% of the ligands are expected to be accessible to the plasmid.

In order to explore binding conditions for pTS2 versus pUC19 we performed binding experiments at different pH values using purified plasmid preparations. Table 1 summarizes the results of a series of binding experiments. Binding decreased with increasing pH for both plasmids over the pH range 4.5–5.5 with pTS2 binding more strongly to the affinity beads than pUC19. These results indicate that triplex affinity purification is best performed at pH 5.0, combining good triplex formation with minimal non-specific binding to the affinity support.

To further explore stringent binding conditions for pTS2 versus pUC19 we performed triplex melting experiments. Plasmids (38 µg pTS2, 47 µg pUC19) were incubated and washed under standard conditions at room temperature and pH 5.0. Temperature was then increased in 5°C increments up to 70°C and unbound plasmid washed out of the column at each temperature with binding buffer. Upon addition of elution buffer, 2.7 µg plasmid pTS2 and 0.8 µg pUC19 were released from the beads. This indicates that while some plasmid was released at increased temperature, both triplexes, the 15mer and the 9mer, have T_m close to or higher than 70°C. Therefore, while pTS2 bound more strongly to the affinity beads under all conditions, it was not possible to find stringent binding conditions for pTS2 versus pUC19 by varying either temperature or pH.

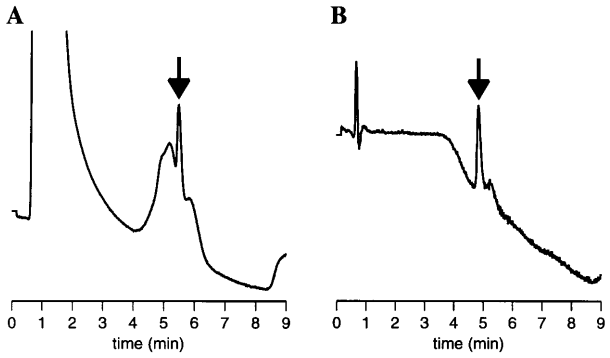

Figure 2. (**A**) HPLC analysis of clarified lysate (RNase-digested). (**B**) HPLC analysis of plasmid DNA (pTS2) after triplex affinity purification. The arrow indicates the plasmid peak. The drift in baseline is due to an increase in salt concentration.

Affinity purification of plasmid pTS2 from clarified lysate

The results described so far were obtained with plasmid preparations purified by conventional methods. Due to its high specificity, it is desirable to apply affinity purification early in downstream processing of plasmid DNA. We therefore investigated triplex affinity purification of plasmid pTS2 from clarified lysate. Clarified lysate of *E.coli* cells was mixed with acetate buffer to give a pH of 5.0 or 4.5 in 2 M NaCl (Materials and Methods). Incubation, washing and elution were performed as described before. Results of two binding experiments at pH 5.0 and 4.5 are shown in Table 1. Similar binding at both pH values and recovery of up to 37% of the loaded plasmid was measured.

HPLC analysis of clarified lysate showed a large, broad peak of high molecular weight RNA overlapping with the plasmid DNA (data not shown), which is characteristic of the Poros column employed (10). For a better representation, we therefore digested a sample of clarified lysate with RNase A (20 µg/ml, 1 h at 37°C). HPLC analysis of RNase-digested lysate (Fig. 2A) showed a large RNA contamination at early elution times and further contamination, most likely cell DNA, which partially overlapped with the plasmid DNA. Plasmid DNA purified by triplex affinity showed only one major peak on HPLC analysis (Fig. 2B). The small peak which elutes shortly after pTS2 in the salt gradient was previously identified as denatured supercoiled plasmid (10). In gel electrophoresis, triplex affinity-purified plasmid appeared predominantly as supercoiled plasmid DNA and no RNA or cell DNA contamination was detectable (Fig. 3).

Total protein concentration in the clarified lysate was 146 µg/ml, which corresponds to 29 µg/µg plasmid. Total protein in the triplex affinity purified plasmid fraction was 1.3 µg/ml (0.68 µg/µg plasmid), which corresponds to a >100-fold reduction per ml and to a 43-fold reduction per µg plasmid.

DISCUSSION

In the present paper we describe the use of a novel large pore affinity support for the purification of plasmid DNA by triplex affinity interaction. Due to its high mechanical stability and protection of the ligand inside macropores this support can be used in a continuous affinity recycle extraction process (CARE, in progress) (24). Unlike affinity chromatographic methods (21), CARE can easily deal with

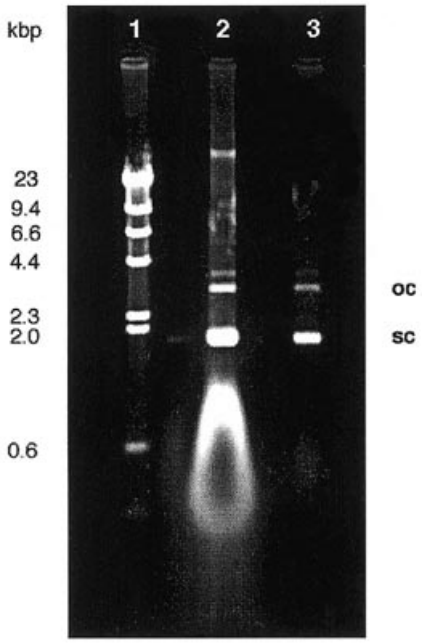

Figure 3. Agarose gel electrophoresis of plasmid pTS2. sc, supercoiled; oc, open circular. Lane 1, λ *Hin*dIII size marker; lane 2, clarified lysate. Cell DNA shows as a smear over the whole path of migration at large DNA sizes, while RNA shows as a large dot running faster than plasmid DNA. Lane 3, plasmid DNA after triplex affinity purification. No cell DNA or RNA is seen.

particulate contaminants and, unlike processes using magnetic beads (20), it can be scaled up to large scale.

Triplex binding experiments showed that efficient binding of plasmid DNA can be achieved at moderately acidic pH with yields of up to 62%. Maximum bead capacity was found to be 28 µg/ml, which is comparable with values published for a small pore triplex affinity support (23–49 µg/ml) (21). While a similar capacity is reported for gel filtration (7), other chromatographic techniques using anion exchange (8,9) or hydroxyapatite (25) columns generally show at least 10-fold higher values. Future development should therefore be aimed at increasing bead capacity by increasing both accessibility of ligands (larger pore size) and ligand density on the support. Special attention should be directed at bead chemistry, as high crosslinking provides the stability necessary for such a support but at the same time is responsible for its low functionalization (T.Schluep and C.L.Cooney, submitted to *J. Chromatogr. A*).

Triplex affinity purification of plasmid DNA from clarified lysate showed efficient removal of RNA, cell DNA and bacterial protein. In HPLC we observed a certain enrichment of denatured supercoiled plasmid, which elutes shortly after the main plasmid peak. This plasmid form is generated by harsh conditions during alkaline lysis, possesses large single-stranded areas and runs at the same speed as supercoiled plasmid in agarose gels (10). It is conceivable that conformational changes induced by denaturation could be responsible for improved triplex formation. In agarose gels we observed that the purified plasmid contained a higher fraction of supercoiled plasmid than the starting material (Fig. 3). While similar observations have been reported previously (21), other authors report the contrary (20), pointing to the possibility that preferential binding might be a function of the target sequence or target position in the plasmid.

In conclusion, triplex affinity purification shows a high potential for downstream processing of plasmid DNA. It is especially suited to production of pharmaceutical grade plasmid preparations as no toxic chemicals or animal-derived enzymes are necessary. Future work will focus on integration of the triplex affinity principle into a continuous affinity purification process which can be easily scaled up. Combination of such a process with classical purification steps will hopefully result in a large scale production process for pharmaceutical grade plasmid DNA.

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