Separation of plasmid DNA from protein and bacterial lipopolysaccharides using displacement chromatography

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Received 3 April 1998; accepted 25 November 1998

Key words: continuous bed column, displacement chromatography, lipopolysaccharide, plasmid DNA, SMA model

Abstract

The preparation of plasmid DNA at large scale constitutes a pressing problem in bioseparation. This paper describes a first investigation of displacement chromatography as a means to separate plasmid DNA (4.7 kb) from *E. coli* lipopolysaccharides and protein (holo transferrin), respectively. Displacement chromatography has advantages in this regard, since the substance mixture is resolved into rectangular zones of the individual components rather than into peaks. Thus a higher total concentration can be maintained in the pooled product fractions. Hydroxyapatite (type I and II) and anion exchange stationary phases were included in the experiments. In addition to a conventional anion exchange column packed with porous particles, the recently introduced continuous bed UNO™ anion exchange column was investigated. No DNA purification was possible with either hydroxyapatite material. Conventional particle based columns in general were not suited to the separation of any two substances varying considerably in molecular mass, e.g. plasmid DNA and standard protein. Presumably, the direct competition for the binding sites, which is essential in displacement chromatography, was restricted by the size dependency of the accessible stationary phase surface area in this case. Better results were obtained with the continuous bed column, in which the adsorptive surface coincides with the walls of the flow through pores. As a result the accessible surface does not vary as much with the size of the interacting molecules as for the conventional stationary phase materials. Sharper transitions were also observed between substance zones recovered from the UNO™ column. The steric mass action model was used to aid method development in case of the anion exchange approach. While further research is obviously necessary, displacement chromatography on continuous bed columns has been shown to be capable of separating plasmid DNA from typical impurities.

Abbreviations: EGTA: Ethyleneglycol-bis(β -aminoethylether)-N,N,N',N'-tetraacetic acid; LAL: Limulus Amebocyte Lysate; LPS: Lipopolysaccharides; PAA: Polyacrylic acid; SMA: Steric mass action

Introduction

The preparation of plasmid DNA at large scale may well become the challenge to bioseparation in the next decade. Large amounts of high-purity plasmid DNA are necessary, e.g., for application in gene therapy, but also for the (transient) transfection of the producing organisms in cell culture technology. The plasmids are routinely produced in *E. coli* and their recovery from the cell lysates may involve precipitation and extraction operations, CsCl₂-density gradient cen-

trifugation as well as various adsorption techniques. By these operations numerous *E. coli* proteins, other polynucleotides, the toxic lipopolysaccharides (LPS) stemming from the cell membrane, etc. are removed. The various conformations of the plasmid, such as supercoiled, nicked, open circular, and linearized molecules are known to differ in transfection efficacy, thus further processing of an already 'pure' batch may sometimes be desirable.

While extraction and precipitation are easily scaled, the resulting product is often of insufficient

Figure 1. Schematic presentation of a chromatographic separation in the elution mode. If some concentration limit is imposed for whatever reason, the entire peak including the maximum must stay below this limit. The overall concentration in the collected peak fraction will be much lower.

quality. The CsCl_2 -density gradient centrifugation yields a highly purified product even in regard to conformation, but it is difficult to use this method at large scale. Adsorption techniques and especially chromatography combine scalability with high resolution and thus present a possibility for large scale applications. DNA is a negatively charged polyelectrolyte, thus anion exchange chromatography may be used for its enrichment. In addition, hydroxyapatite has long since been known to interact strongly with the phosphate backbone of DNA (Bernardi, 1971).

Concentrated DNA-solutions are very viscous. In order to avoid problems such as viscous fingering, exceeding the pressure limit of the system, etc. certain concentration limits may have to be observed. In the well known elution chromatography, the substance mixture is (ideally) resolved into individual 'peaks', Figure 1. In this case the peak maximum would obviously have to stay below the concentration limit and thus the average concentration of the peak volume would be quite low. In displacement chromatography, on the other hand, the substances are focused into consecutive zones of a constant concentration by an advancing displacer front, Figure 2 (Antia and Horvath, 1989). The concentration in the zones can be controlled via the displacer concentration and thus be kept in its entirety just below the critical value. The overall concentration in the pooled product fractions may thus be considerably higher than in the elution mode, while smaller columns can be used to give the same amount of product due to a more effective use of the column's capacity (Kasper et al., 1996).

The design of a successful displacement separation may be more difficult than that of an elution separation. The displacer, e.g., should be a 'substance which shows a superior binding to the stationary phase under operating conditions'. This is not an easy requirement to fulfill in the case of DNA, which tends to interact strongly with both anion exchange and hydroxyapatite stationary phases. In this paper we want to report on first experiments concerning the separation of plasmid DNA from both proteins and *E. coli* lipopolysaccharides using the displacement approach.

Materials and methods

Materials

Proteins, fine chemicals including ethyleneglycol-bis(β -aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), as well as the bulk chemicals for buffer and eluent preparation were from Sigma, Deisenhofen, Germany. Polyacrylic acid (PAA, average molecular mass: 5100 g mol^{-1}) was from Fluka, Neu-Ulm, Germany. High purity water and chemicals of analytical grade quality were used throughout. Holo-transferrin and *E. coli* LPS (serotype 026:B6, Lot 46H4024) were from Sigma, Deisenhofen, Germany. A LAL (limulus amoebocyte lysate) assay kit for LPS quantification was obtained from Pyroquant Diagnostik GmbH, Walldorf, Germany.

The following stationary phases and columns were supplied by Bio-Rad, Hercules, CA, U.S.A. and used for displacement chromatography: a 2 mL BioScale Q2 strong anion exchanger column ($7 \times 52 \text{ mm}$, packed with porous, $10 \mu\text{m}$ particles), a 1.3 mL UNO™ Q1 continuous bed strong anion exchanger column ($7 \times 35 \text{ mm}$), and two columns ($250 \times 4 \text{ mm}$) filled with porous, ceramic hydroxyapatite beads ($10 \mu\text{m}$, type I and type II). For the RPC-analysis of the displacement fractions, a Hytack C18 column ($4.6 \times 30 \text{ mm}$) (Glycotech, U.S.A.) filled with non-porous particles was used.

Plasmid preparation

Plasmids (pEGFP-N1, Clontech, Palo Alto, California, U.S.A.) were produced in *E. coli* (DH5 α) grown in LB medium (Gibco Life Technologies Inc., Gaithersburg, Maryland, U.S.A.) containing $50 \mu\text{g mL}^{-1}$ kanamycin. Qiagen (Basel, Switzerland) kits were used for plasmid isolation according to the manufacturer's instructions.

Figure 2. Schematic presentation of a chromatographic separation in the displacement mode. The substances appear as zones rather than peaks and the zone concentration can be kept just below the imposed maximum. The overall concentration in the pooled product fractions will thus be close to the optimum value.

Displacement chromatography

The displacement system was assembled from an ERC HPLC pump 64 (ERC, Alteglofsheim, Germany) and a Valco 10-port valve (Valco, Houston, Texas, U.S.A.). A 1 mL loop was used for sample injection, the displacer was introduced from a 5 mL preparative sample loop (Knauer, Berlin, Germany). A flow rate of 0.1 mL min^{-1} was used throughout. Column regeneration was by a high salt step (2 M NaCl) in all cases. Fractions were collected twice per minute (average fraction volume $50 \mu\text{L}$). Both EGTA and PAA were used as displacers.

Analytical methods

Protein concentrations in solution were measured either by the BCA assay (Pierce) according to the manufacturers instruction or by reversed phase chromatography (RPC) as described previously (Breier and Freitag, 1995). Plasmid DNA in the displacement fractions was quantified as described by Sten et al. (1993). LPS concentrations were measured either

by the kinetic LAL assay according to the manufacturer's instructions or by capillary electrophoresis as described previously (Freitag et al., 1997).

Isotherm measurements

The system for the isotherm measurements consisted of an ERC HPLC pump 64 (ERC, Alteglofsheim, Germany) and a Valco 10-port valve (Valco, Houston, Texas, U.S.A.). Isotherms were measured by recording breakthrough curves for different concentrations. A 0.02 M TRIS/HCl buffer pH 8 was used as mobile phase. The flow rate was 0.5 mL min^{-1} in all cases. The UNOTM Q1 column was used as stationary phase.

SMA parameters

Unless indicated otherwise, the SMA parameters, the affinity plot and the operating regime plot were calculated as outlined by Kundu et al. (1997). The characteristic dimensionless charge, ν , was calculated from conductivity measurements. The equilibrium constants, K , were calculated from the linearized

Figure 3. Displacement separation of a protein/DNA mixture. The chromatographic conditions were as follows: Column dimensions: 250×4 mm, stationary phase: porous HA type I ($10 \mu\text{m}$ average particle diameter), carrier: 0.005 M phosphate pH 7.2, displacer: EGTA.

Figure 4. Displacement separation of a DNA/lipopolysaccharide mixture. The chromatographic conditions were as follows: Column dimensions: 250×4 mm, stationary phase: porous HA type II ($10 \mu\text{m}$ average particle diameter), carrier: 0.005 M phosphate pH 7.2, displacer: EGTA.

Figure 5. Displacement separation of a protein/DNA mixture. The chromatographic conditions were as follows: Column dimensions: 250×4 mm, stationary phase: porous HA type II ($10 \mu\text{m}$ average particle diameter), carrier: 0.005 M phosphate pH 7.2, displacer: EGTA.

isotherms (assuming the Langmuir equation to apply). The Δ -point, corresponding to Q_D/C_D (with Q_D : stationary phase concentration of the displacer (mM mL^{-1} stationary phase), C_D : mobile phase displacer concentration (mM mL^{-1})), was taken from the displacer isotherm. The affinity plot was then created by first defining the $\log K$, ν -points for all substances and then connecting the respective individual

points with the Δ -point. A comparison of the slopes of the resulting lines allowed a statement concerning the possibility of displacement and the order of appearance of the substances in the displacement train. The operating regime plot was created by calculating the displacement and the elution line, i.e. for a given DNA molecule, Δ point, and mobile phase salt concentration, the minimum (displacement line) and the maximum (elution line) displacer concentration, which will still allow true displacement to take place. A lower displacer concentration will result in an elu-

Figure 6. Comparison of plasmid DNA fractionation in the elution mode using a fixed bed and a continuous bed anion exchange column. Conditions were: stationary phase (peak 1, full line): BioScale Q2 column (7×52 mm, $10 \mu\text{m}$ porous particles) and (peak 2, broken line): UNOTM Q1 (7×35 mm), flow rate: 0.5 mL min^{-1} , sample: $10 \mu\text{L}$ (corresponding to $2 \mu\text{g}$ of plasmid DNA), gradient: 0–5 min 100% buffer A (20 mM TRIS/HCl, pH 7.4), 5–10 min 100% buffer A to 100% buffer B ((20 mM TRIS/HCl, pH 7.4 + 1 M NaCl), 10–12 min 100% buffer B, 12–13 min 100% buffer B to 100% buffer A, 13–18 min 100% buffer A.

Figure 7. Single component isotherms. Conditions: Buffer: 0.02 M TRIS/HCl pH 8, flow rate: 0.5 mL min^{-1} , stationary phase: UNOTM Q1.

tion of the substance (here the plasmid DNA) within the displacer zone, while a higher displacer concentration will result in an elution of the plasmid DNA ahead of the displacer zone as a result of the induced salt step (Brooks and Cramer, 1992).

Figure 8. Dynamic affinity plot for the separation of plasmid DNA and *E. coli* LPS aggregates (assumed molecular weight 80 kg mol^{-1}) by polyacrylic acid (PAA, Mw: 5100 g mol^{-1}).

Results and discussion

The purification of plasmid DNA from *E. coli* cell lysates involves the removal of proteins, other polynucleotides (genomic DNA, RNA, etc.), and lipopolysaccharides. Residual LPS does present a major problem since it is a known disadvantageous agent in both medical and cell culture technology application. Just as DNA, the LPS molecules bear negative charges at

Figure 9. Operating regime plot for the plasmid DNA displacement by polyacrylic acid.

high charge density, so both types of molecule interact strongly with anion exchange phases. Since both molecules typically contain phosphate groups, interaction with hydroxyapatite is also possible in both cases. The removal of certain acidic proteins may also present a problem.

Hydroxyapatite plasmid DNA chromatography

Hydroxyapatite (HA) is a crystalline calciumphosphate modification with known affinity to molecules containing phosphate groups. DNA preparation has been among the earliest applications suggested for the material (Bernardi, 1972). However, together with the phosphate binding C-sites, the HA surface does also bear a negative net charge (P-sites). Negatively charged substances such as DNA will thus also be repulsed by the surface and the surface chemistry (P-site/C-site ratio) will influence the binding to a large degree. Bio-Rad offers two types of HA, type I for protein applications and type II for DNA applications. According to the manufacturer the DNA capacity of the type II material is three times higher than that of type I. Both types were investigated by us for the separation of DNA from sample containing either a mixture of DNA and LPS or of DNA and protein (holo transferrin). EGTA (Ethylenglycol-bis(β -aminoethylether)-N,N,N',N'-tetraacetic acid), a substance that in the past had given good results as displacer of C-site interacting substances (Vogt and Freitag, 1997) was used as displacer.

No displacement separation was possible in case of the type I HA. DNA and LPS were strongly retained on the column, while the majority of the protein eluted together with traces of the DNA in the displacer zone, Figure 3. With the type II material, a displacement of DNA and LPS by EGTA was possible, however, no separation took place, since both substances were found in all fractions at roughly the same concentra-

tion ratio, Figure 4. A change in the displacement conditions (displacer concentration, salt concentration and pH of the carrier) within the range defined by the necessity to maintain displacement conditions as well as the integrity of the stationary phase and the chromatographic system, did not improve the separation. It is possible that this inability to separate is the result of an elution azeotrop, i.e. to due a pronounced similarity of the binding strength of both substances to the stationary phase (Kasper et al., 1996).

A different problem was encountered during the attempts to separate the standard protein from the DNA (or likewise from the LPS). In such mixtures the DNA (respectively the LPS) was displaced and appeared in a roughly rectangular zone in front of the displacer. The protein appeared in a peak shaped zone that was superimposed on the DNA or LPS zone. Figure 5 illustrates this taking a separation between LPS and the standard protein as example. We interpret this as the direct result of a pronounced difference in size between the protein on one side and the DNA (4.7 kb), respectively, the LPS aggregates (M_w 80 kg mol⁻¹ according to light scattering measurements) on the other. Thus, the inner particle surface is almost inaccessible to the larger molecules, while the proteins can be adsorbed there. As a consequence the direct competition for the binding site, a necessity for the development of well-defined zones, is not taking place between protein and DNA molecules (LPS aggregates).

Conventional stationary phases (porous particles)

A similar problem in separating molecules of widely different size was consequently also observed in the case of the BioScale Q2 column packed with porous strong anion exchanger beads (10 μ m). The separation of protein and DNA (LPS aggregates) was not possible. Plasmid DNA and LPS aggregates seemed to be of more equal size and did compete for binding. Their separation was possible, but the overlap between the zones was broad, presumably also due to pronounced mass transfer limitations.

Continuous bed columns (UNOTM Q column)

Mass transfer in general tends to present a problem in the isolation of biopolymers with columns packed from conventional, i.e. porous, stationary phase particles. The major part of the adsorptive surface in the pores can only be reached by molecular diffusion, a time consuming task for large biopolymers. The prob-

Figure 10. Displacement of the pure substances from the UNO™ Q1 column. a) DNA; b) LPS; c) the standard protein. The chromatographic conditions were as follows: Column dimensions: 7 × 35 mm, carrier: 0.02 M TRIS/HCl pH 8.0, displacer: PAA.

Figure 11. Displacement separation of a DNA/lipopolysaccharide mixture. The chromatographic conditions were as follows: Column dimensions: 7×35 mm, carrier: 0.02 M TRIS/HCl pH 8.0, displacer: PAA.

lem exists for all types of biopolymer chromatography, not only in the displacement mode.

Recently, a special stationary phase, namely the continuous bed UNOTM column (Bio-Rad), has been introduced in the hope to circumvent the problem. The UNOTM column is a polymer rod prepared in situ by radical polymerization. It consists of a continuous network of polymer nodules with interconnecting channels. Thus the surface is high, while there is no 'intraparticle' surface. Band broadening is small even at high flow rates, as we were recently able to show (Vogt and Freitag, 1998).

The surface chemistry and thus the interaction mode of the UNOTM Q column is similar to that of the comparable conventional anion exchanger column (BioScale Q) from the same manufacturer. However, a comparison of a plasmid DNA fractionation in the elution mode (flow rate 0.5 mL min^{-1}) already shows differences between the two anion exchangers, Figure 6. The resulting peak is much broader in case of the packed bed column. Even though the linear flow rate was the same in both cases, the retention time is higher for the continuous bed column. A possible explanation can again be based on the supposed differences in the accessible surface area. The ratio between accessible and total surface area will in the case of biopolymers be more favorable for the continuous bed column.

By definition, displacement chromatography occurs under non-linear adsorption conditions. The design of a successful displacement separation can thus be difficult, especially in the case of HA-chromatography where the interaction is not governed in a first approximation by a single parameter (e.g.

the salt concentration of the carrier) but by several such parameters. At present no model exists for non-linear HA-chromatography. In case of ion exchange chromatography, the SMA model has in the past been used successfully for the design of protein and peptide separations in the displacement mode. Here the model is for the first time applied to the separation of large and highly charged molecules such as DNA and LPS (aggregates).

A first problem was encountered during the determination of some of the SMA parameters, e.g. the affinity constant, K . Normally this is determined by isocratic elution experiments. This is not possible for DNA and LPS molecules. A calculation of this value from the linearized (Langmuir) isotherms yielded data that were in agreement with the experimental results. The assumption of a molecular weight of only $20\,000 \text{ g mol}^{-1}$ for the *E. coli* LPS also could not be brought into alignment with some of the experimental findings. Light scattering experiments with LPS in a solution approximating the carrier showed that the majority of the LPS molecules was found in stable aggregates with an average molecular mass of $80\,000 \text{ g mol}^{-1}$. Assuming this 'molecular mass', the isotherms shown in Figure 7 were calculated. Due to the size of the molecule, the DNA isotherm is difficult to see, if extrapolated to higher carrier concentrations, however, it would be below the other isotherms. It can also be deduced, that polyacrylic acid (PAA, Mw 5100 g mol^{-1}) is a possible displacer of all involved substances.

The expected order in the displacement train is DNA, LPS, protein, followed by the displacer front. This conclusion was corroborated by the dynamic affinity plot, where the slope is steepest for DNA followed by the LPS and the displacer, Figure 8. The ability of PAA to displace proteins from the UNOTM column has already been demonstrated (Vogt and Freitag, 1998). The operating regime plot can be helpful in defining buffer salt/displacer concentrations conducive to displacement. Figure 9 demonstrates that in a first approximation, the displacer concentration to be used in combination with a carrier salt concentration of 20 mM should be at least 13 mM to avoid elution of the DNA in the displacer zone, and no more than 39 mM to avoid the premature elution of the DNA in the salt step induced by the advancing displacer front.

The predictions of the model corresponded to the experimental results. The displacement of all three substance classes by PAA was possible, Figure 10. The shape of the substance zones was rectangu-

lar and the concentration increased in the expected order. Experiments with two component mixtures (DNA/protein, DNA/LPS, protein/LPS) pointed into the desired direction. Compared to the result obtained with the fixed bed anion exchanger column, the overlap between the zones was less pronounced. For the first time a separation between the DNA and the LPS seemed possible, Figure 11.

Conclusion

The isolation of plasmid DNA at larger scale continuous to present a problem in preparative biotechnology. Given both its scalability and its high resolution, chromatographic operations and especially displacement chromatography may well contribute to the final answer to this problem. However, the results are far from satisfactory and much further research including among other things an investigation of the transfection efficiency to prove that the procedure does not affect the biological activity will be necessary before displacement chromatography can become a viable option in DNA preparation.

Acknowledgments

We would like to thank Ms. Tiziana Flego and Ms. Alexandra Kulangara for the preparation of the plasmids. Mr. Roberto Giovannini carried out the elution chromatography experiments. All three also contributed in the area of plasmid analysis.

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