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Polyelectrolyte complexes as a tool for purification of plasmid DNA Background and development

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Abstract

The demand for highly purified plasmids in gene therapy and plasmid-based vaccines requires large-scale production of pharmaceutical-grade plasmid. Plasmid DNA was selectively precipitated from a clarified alkaline lysate using the polycation poly(*N*,*N*′-dimethyldiallylammonium) chloride which formed insoluble polyelectrolyte complex (PEC) with the plasmid DNA. Soluble PECs of DNA with polycations have earlier been used for cell transformation, but now the focus has been on insoluble PECs. Both DNA and RNA form stable PECs with synthetic polycations. However, it was possible to find a range of salt concentration where plasmid DNA was quantitatively precipitated whereas RNA remained in solution. The precipitated plasmid DNA was resolubilised at high salt concentration and the polycation was removed by gel-filtration.

Keywords: Purification; Plasmid DNA; Polyelectrolyte complexes

1. Introduction

In the past decade, gene therapy has become a reality and clinical trials have begun for preventing or treating diseases, e.g. cystic fibrosis, cancer and AIDS [1]. Both viral [2,3] and non-viral vectors [4] have been used for delivery of nucleic acids into target cells. Non-viral vectors are regarded safer and have become an increasingly desirable alternative [4,5]. Non-viral vectors based on plasmid DNA are, on the other hand, less effective at transfecting target cells than viral vectors why large amounts of plasmid will be required. Plasmid DNA has also been used for vaccination by stimulating and enhancing immune system response [6–8]. The demand for highly purified plasmids in gene therapy and plasmid-based vaccines thus requires large-scale production of pharmaceutical-grade plasmid.

The large-scale purification of plasmid DNA from bacterial cell culture normally includes one or several chromatographic steps [9]. The application of different chromatography techniques such as anion-exchange, size-exclusion, hydrophobic interaction and triple helix affinity chromatography have been reviewed elsewhere [9]. Capture and pre-column purification has been performed on a small-scale for molecular biology-based methods by extraction with laboratory protocols including relatively large volumes of organic solvents. A major disadvantage using this kind of chemicals is that most of the organic solvents are toxic and flammable and require special industrial facilities. Precipitation of plasmid DNA by polyethylene glycol/salt systems or by polyethylene glycol alone have been described as more suitable methods and are preferred to precipitation by alcohols or organic solvents [10]. After concentration of plasmid by precipitation further purification can be performed by another precipitation step, using salts like, e.g. ammonium sulphate [11]. Recently, more selective precipitation techniques have also been reported, spermidine [12], cetyltrimethylammonium bromide (CTAB) [13] and affinity precipitation based on triple helix formation [14]. Although none of these precipitation techniques is working directly on a clarified lysate prepared by alkaline lysis.

Polycations was earlier introduced for the formation of soluble complexes used in non-viral gene delivery [15,16]. The formation of insoluble polyelectrolyte complexes

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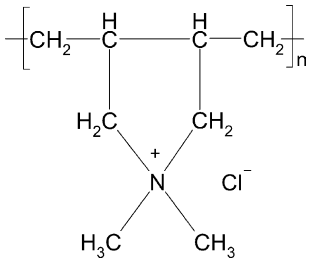

Fig. 1. Structure of the polycation poly(*N*,*N*′-dimethyldiallylammonium) chloride (PDMDAAC).

between polycations and nucleic acids has been recently studied by us [17]. We here demonstrate that precipitation of plasmid DNA, as capture step from a clarified alkaline lysate, can be achieved by the addition of the polycation poly(*N*,*N*′-dimethyldiallylammonium) chloride (PDMDAAC) (Fig. 1). PDMDAAC is a commercially available polymer approved for use as flocculant in potable water [18].

2. Experimental

2.1. Materials

2.1.1. Nucleic acids

RNA from bakers yeast was purchased from Sigma (St. Louis, MO, USA). Frozen cell paste (−80 °C, dry weight 0.22 g/g cell paste) of *Escherichia coli* XL1 Blue harbouring the plasmid pBluescript II KS (±) 2.9 kbp having an insert of a xylanase gene from *Rhodothermus marinus* (3 kbp) giving a total plasmid size of 5.9 kbp was obtained from The Swedish Centre for Bioseparation. A pure plasmid preparation pJV4, consisting of pUC 19 (2.7 kbp) having a 3.4 kbp insert (JV4–dmgA–demA gene) from Streptococcus dysgalactiae giving a total plasmid size of 6.1 kbp was a kind gift from Amersham Biosciences, Uppsala, Sweden. Supercoiled DNA ladder (2067–16210 bp) was obtained from Invitrogen Co. (Carlsbad, CA, USA).

2.1.2. Polyelectrolytes

Poly(*N*,*N*′-dimethyldiallylammonium) chloride with an average degree of polymerization (DP) of 1400 was purchased from CPS Chemical Company (Arkansas, USA), while poly(sodium-*p*-styrenesulfonate) (PSS) with an average DP of 340 was obtained from Acros Organics (New Jersey, USA).

Zeolite Y, with a SiO_2/Al_2O_3 mole ratio of 430, was obtained from Tosoh Co. (Japan). Bicinchoninic acid (BCA), bovine serum albumin (BSA) and copper sulphate for bicinchoninic acid protein assay, Sigma procedure no. TPRO-562, as well as RNase A were obtained from Sigma. Salts and buffers used in all experiments were of analytical grade.

2.2. Analysis

2.2.1. Size-exclusion chromatography

Sephacryl S-500 beads was packed into a XK 16/20 column, which was connected to an ÄKTA^{TM} explorer chromatography system (all obtained from Amersham Biosciences, Uppsala, Sweden). The column, with a final bed volume of 22 ml, was equilibrated with 2 M KAc pH 5.5 or 25 mM Tris–HCl, pH 8 containing 2 M NaCl. One millileter samples (if nothing else is mentioned) were injected and run at a flow rate of 1 ml/min (30 cm/h). The eluted peaks were detected at 260 and 280 nm.

2.2.2. Ion-exchange chromatography

The recovery of supercoiled plasmid was determined by analytical ion-exchange chromatography on a MiniQ column (4.6/50 mm) integrated to an ÄKTA^{TM} explorer chromatography system (all obtained form Amersham Biosciences, Uppsala, Sweden) and equilibrated with 25 mM Tris–HCl, pH 8 containing 0.5 M NaCl. To avoid the interference of RNA in the quantitation of plasmid, all samples were treated with RNase. Samples (100 μl) were injected and subsequent application of a gradient from 0.5 to 0.8 M NaCl in 18 column volumes eluted the nucleic acids. The eluate from the column was monitored by UV absorbance at 260 and 280 nm. The analysis was performed at a flow rate of 0.4 ml/min.

2.2.3. Protein determination

The samples were assayed for protein concentration using the BCA method, Sigma procedure no. TPRO-562. The samples were left in the BCA assay for 1 h in darkness at room temperature and the absorbance at 562 nm was determined. Bovine serum albumin was used as a standard.

2.2.4. Gel electrophoresis

Gel electrophoresis was performed on 0.7% agarose gels in TBE buffer (0.089 M Tris-borate, pH 8.0, 2 mM EDTA). Fifteen microliter (including 2.5 μl of glycerol 50%) samples was loaded in each well and the samples were run at 60 V for 60 min on a Hoefer HE 33 Mini horizontal submarine unit (Amersham Biosciences) powered by a electrophoresis power supply (EPS 301, Amersham Biosciences). After the run the agarose gel was stained with ethidium bromide by soaking the gel in 100 ml TBE buffer containing 1.5 μg ethidium bromide per ml. The agarose gel was analysed and photographed using the gel documentation software AlphaImager 2200 v5.5 from Alpha Innotech Corporation (San Leandro, CA, USA).

2.3. Preparation of pure plasmid DNA for model precipitation experiments

The plasmid DNA was purified by the Qiagen Plasmid Mega kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer instruction.

2.4. Precipitation of nucleic acids

Solutions of nucleic acid, potassium acetate (KAc) and polycation were mixed and shaken vigorously for at least 1 min and then centrifuged at 14,100 × *g* for 10 min. The absorbance at 260 nm was then determined in the supernatants. The polycation, PDMDAAC, did not absorb light at 260 nm and hence did not hinder the assay. Data obtained are presented in terms of % of remaining nucleic acid after precipitation, i.e. the absorbance remaining in the supernatant divided by the absorbance of the initial nucleic acid solution under the same conditions. Calculation of charge density of nucleic acids, i.e. the concentration of phosphate groups [P], was determined indirectly by UV absorbance measurements at 260 nm, assuming an average nucleotide molar absorption coefficient of 6500 l mol^{-1} cm^{-1} [19]. The composition of polycation–nucleic acid mixtures was expressed as the charge ratio [+]/[−] = [quaternized amino groups of the polycation]/[P].

2.5. Removal of SDS from clarified alkaline lysate

Zeolite suspension (160 mg/ml) was prepared by mixing solid Zeolite Y with Tris–HCl and NaCl. The final concentration of Tris–HCl was 2 mM and NaCl concentration was 0.2 M. The suspension was incubated at room temperature during gentle mixing for 20 min. Zeolite suspension (16 mg zeolite per mg SDS [20]) was then added to a clarified lysate, prepared as described above, mixed for 60 s and then centrifuged for 10 min at 13,000 × *g* at 4 °C. The supernatant was collected and stored in refrigerator until used.

2.6. Precipitation of plasmid DNA from clarified alkaline lysate

Clarified alkaline lysate was prepared according to Horn et al. [21]. The lysate (105 ml) was treated with zeolite to remove SDS as described above. To this zeolite-treated lysate 11.1 ml 2 mM PDMDAAC was added and the final volume was set to 210 ml by the addition of water. The sample was mixed for about 60 s and centrifuged for 10 min at 14,100 × *g* (15–20 °C). After decanting the supernatant, the pellet was re-dissolved in 5 ml 25 mM Tris–HCl, pH 8 including 2 M NaCl. Size-exclusion chromatography was performed to determine the content of plasmid DNA and RNA while BCA was used for proteins. The clarified lysate, the zeolite-treated lysate and the re-dissolved pellet were analysed by these methods. Analytical ion-exchange chromatography (MiniQ) was performed on the clarified lysate and on a fraction of the plasmid peak from the re-dissolved pellet, which was collected from the size-exclusion chromatography.

2.7. Removal of polycation by size-exclusion chromatography

A sample consisting of 0.032 mg/ml plasmid DNA (pBluescript, 5.9 kbp), 25 mM Tris–HCl, pH 8, 0.14 M NaCl and 0.4 mM polycation was prepared. The sample was mixed and shaken vigorously for 1 min and then centrifuged at 14,100 × *g* for 10 min. After removal of the supernatant, the pellet was re-dissolved in 0.2 ml 25 mM Tris–HCl, pH 8 containing 2 M NaCl. The re-dissolved pellet (0.1 ml) was then applied on a XK 16/20 column (11 cm × 1.6 cm) packed with Sephacryl S-500 beads and integrated to an ÄKTA^{TM} explorer system (all obtained from Amersham Biosciences, Uppsala, Sweden). The column was equilibrated with 25 mM Tris–HCl, pH 8 containing 2 M NaCl and the flow rate was 0.5 ml/min. Fractions of 1 ml were collected. Detection of the polycation PDMDAAC was performed by titrating one fraction at a time with poly(sodium-*p*-styrenesulfonate). The formation of a insoluble polyelectrolyte complexes between PDMDAAC and PSS could then be detected by turbidity measurements. After adding a small volume of 2 mM PSS and mixing, the turbidity was taken as the absorbance at 350 nm. The procedure was repeated until maximum in turbidity was obtained. Since the PSS solution gave a very small contribution to absorbance at 350 nm, the detection limit for the turbidity was set to 0.01 AU, i.e. only $A_{350} \geq 0.01$ was regarded as turbidity due to formation of insoluble PECs.

3. Results and discussion

3.1. Polyelectrolyte complexes: a short theoretical background

Polyelectrolyte complexes are the products of the interaction between oppositely charged polyelectrolytes. PECs are affected by the properties of the interacting polyelectrolytes (e.g. charge density [22] and chain length [23]) and the chemical environment (e.g. ionic strength [24,25]). When a complex is formed the counter ions belonging to the polyelectrolytes are released. Thus, entropy is the driving force in the formation of PECs. The simplest way to prepare PECs is by mixing two aqueous solutions containing the polyanion and the polycation, respectively.

The cooperative multi-site interaction between the polyelectrolytes provides high stability with respect to dissociation of the complex back to the original polyelectrolyte components. The dissociation constant decreases sharply with increasing chain length and reaches nearly zero, even for rather low DP of the polyelectrolytes [26]. The stability of PECs is dependent on the ionic strength, but also the nature of the salt added [27]. The solubility of the PECs depends on the ratio of the charges of the oppositely charged ionic groups, on the relative length of the oppositely charged polyelectrolytes and the ionic strength. Insoluble PECs are always obtained when the net charge of the complex is zero. Negatively or positively charged soluble non-stoichiometric PECs (NPECs) consist of one longer hydrophilizing polyelectrolyte and several shorter oppositely charged

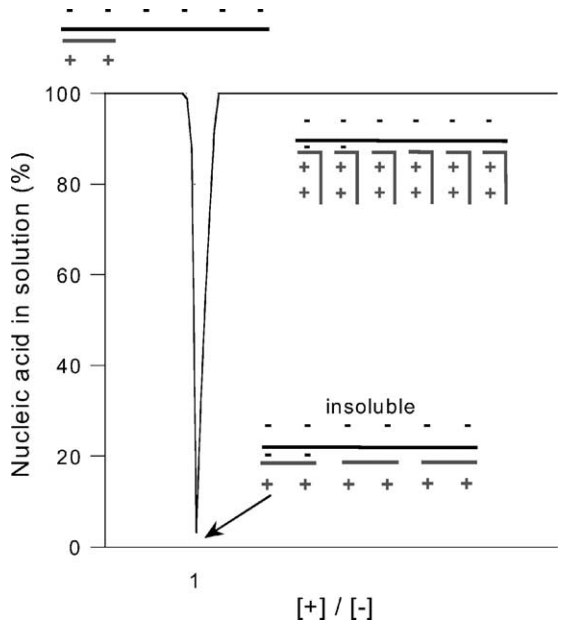

Fig. 2. The formation of PECs in the absence of salt, schematically presented.

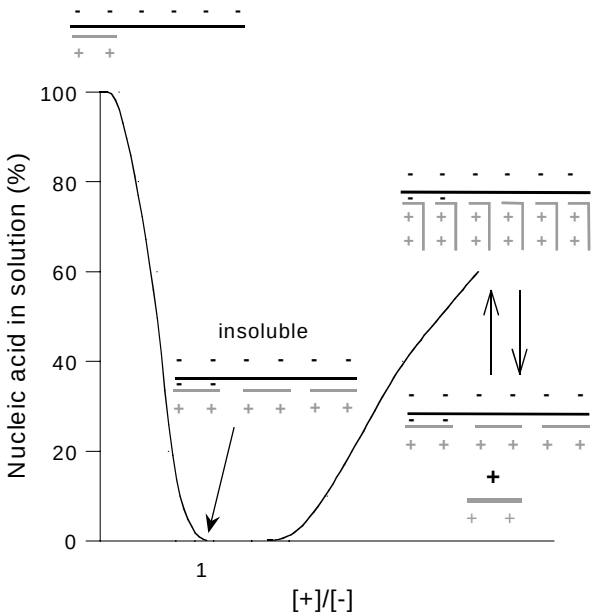

Fig. 3. The formation of PECs in the presence of a moderate salt concentration, schematically presented.

polyelectrolytes [26,28]. Solubility is provided via the hydrophilic single-stranded segments of the complex.

3.2. PECs formed with nucleic acids

Polyelectrolyte complexes are formed by charge–charge interactions between the nucleic acid and the polycation. Precipitation due to formation of insoluble PECs occurs, in the absence of salt, when all charges of the nucleic acid are neutralized by the charges from the polycations (i.e. when the charge ratio $\varphi = [+]/[-] = [\text{quaternized amino groups of the polycation}]/[\text{P}] = 1$). At low values of φ, water-soluble NPECs are formed in the absence of salt (Fig. 2). When a certain critical φ value ($\varphi_{\text{critical}}$) is attained, precipitation of the nucleic acid is initiated. Further addition of the polycation will lead to complete precipitation at $\varphi = 1$, due to equal numbers of positive and negative charges on the PECs. If a minor excess of polycation (positive charges) is added compared to nucleic acid (negative charges), the PECs will become soluble again. This is due to the formation of positively charged PECs. The excess polycation chains added are incorporated into the PECs and act as solubilizing tails. It should be noted that in the case of DNA, no soluble PECs are formed at $\varphi_{\text{critical}} < \varphi < 1$, since the maximum precipitation of DNA coincides with the amount of polycation added [29]. The addition of salt broadens the range of φ corresponding to the formation of insoluble PECs (Fig. 3). This broadening is mainly due to the prevention of the formation of positively charged soluble PECs by the addition of salt, i.e. the equilibrium in Fig. 4 is shifted to the left side. Upon the addition of high concentrations of salt the insoluble PECs start to dissolve due to competitive binding of Na^+ to DNA [23]. Thus, the addition of salt prevents first the formation of positively charged PECs (at $\varphi > 1$) by screening the charges of the additional polycations. But when the salt concentration reaches a certain value, the non-polymeric cations start to interact with the DNA. The salt concentration needed for dissolving the PECs depends on the charge ratio φ.

3.3. Precipitation behaviour of different nucleic acids

In general, phase separation in mixtures of solutions of polycations and nucleic acids follows the general rules determined for synthetic PECs. However, the specific structure of nucleic acids seems to be the reason for some differences in the behaviour of these PECs. The double helix of DNA appears to be responsible for the revealed difference in the phase diagrams of PECs formed by synthetic polyanions and those formed by DNA [29]. The type of polycation (integral or pendant) and the type of counter ion, as well as the DP of the polycation and the DP of the nucleic acid were found to affect the precipitation efficiency [17,29].

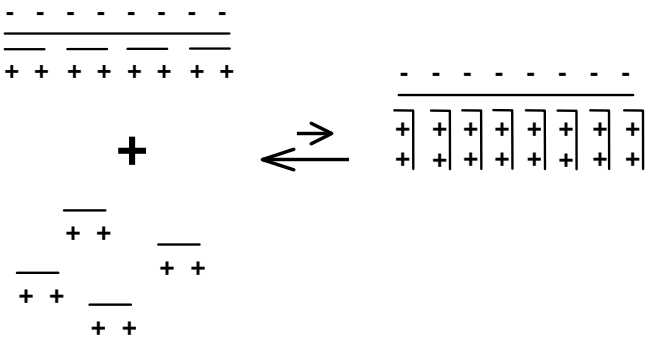

Fig. 4. The equilibrium between soluble and insoluble PECs.

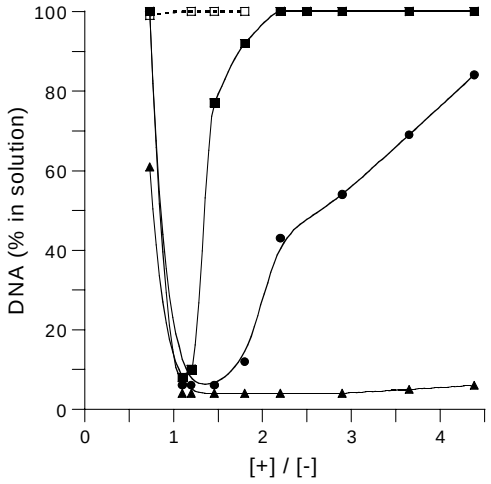

Fig. 5. The relative residual absorbance of DNA remaining in solution after precipitation with PDMDAAC as a function of the charge ratio, [+]/[−]. Concentrations of NaCl were (M): 0 (closed squares); 0.04 (closed circles); 0.12 (closed triangles) and 0.9 (open squares). Representative results of three to four measurements.

In the presence of salt the precipitation was complete even at [+]/[−] > 1. The efficiency of the precipitation by the polycation increased gradually with increasing salt concentration, and in the case of DNA the complete precipitation was obtained at 0.12 M NaCl (Fig. 5). The double helix of DNA was also shown to form much more stable PECs than RNA (see Fig. 6). The PDMDAAC-induced precipitation behaviour of highly polymerised DNA and RNA in the presence of sodium chloride indicated that DNA could be specifically precipitated from the mixture in the presence of 0.5 M NaCl [17]. These findings formed the basis for the development of a precipitation procedure used for the capture of plasmid DNA from a clarified alkaline lysate.

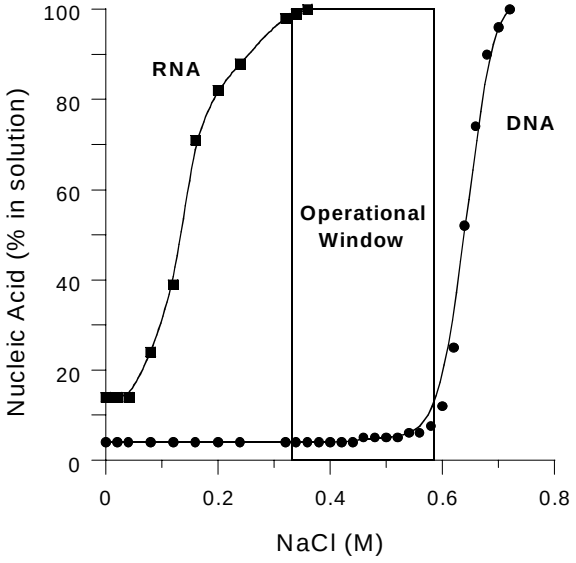

Fig. 6. The relative residual absorbance of RNA (closed squares) and DNA (closed circles) remaining in solution after precipitation PDMDAAC as a function of NaCl concentration at charge ratio, [+]/[−], corresponding to maximum precipitation. Representative results of three to four measurements.

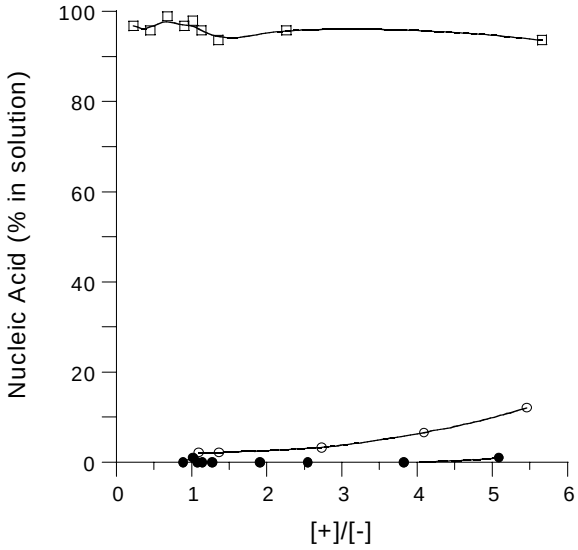

Fig. 7. The relative residual absorbance of different nucleic acids remaining in solution after precipitation with PDMDAAC at 1 M potassium acetate as a function of the charge ratio, [+]/[−]. Nucleic acids: pBluescript (closed circles), pJV4 (open circles) and RNA (open squares). Representative results of three to four measurements.

In a clarified alkaline lysis, used for disrupting cells in plasmid purification protocols, the dominating salt is potassium acetate. PDMDAAC-induced precipitation of RNA and two purified plasmids (pBluescript 5.9 kbp and pJV4 6.1 kbp) at different concentrations of potassium acetate (data not shown) showed that DNA formed stronger complexes than RNA. Both plasmids were completely precipitated in the presence of 1 M KAc, whereas RNA remained in a soluble form (Fig. 7).

3.4. Precipitation of plasmid DNA from a clarified alkaline lysate

In a clarified lysate prepared by alkaline lysis, RNA is by far the major contaminant. RNA interferes significantly in the purification of plasmid DNA, i.e. decreases the capacity of following chromatographic steps. The RNA should thus be removed in the initial pre-chromatographic steps. Consequently RNase has been used in several purification protocols. The use of RNase could make the validation of the process problematic and thus the use of enzymes in the purification of plasmids should be avoided [30].

The results obtained above, i.e. the complete precipitation found in a broad [+]/[−]-region at 1 M KAc, implied the possibility to “over-titrate” the plasmid DNA with the polycation. This provides the precipitation procedure with a wide operational window with regards to polycation and salt concentrations. As a consequence the procedure is less sensitive to changes in the concentration of plasmid DNA, i.e. to batch-to-batch variations in plasmid concentration in the

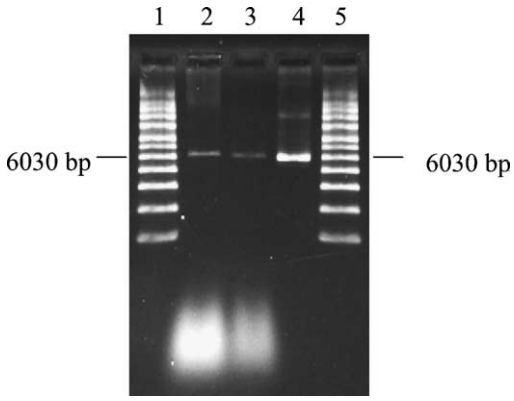

Fig. 8. Agarose gel electrophoresis of samples in the course the plasmid DNA precipitation from clarified lysate. Lanes 1 and 5: supercoiled DNA ladder; Lane 2: clarified lysate; Lane 3: zeolite-treated clarified lysate; Lane 4: re-dissolved pellet (after size-exclusion chromatography).

clarified alkaline lysate. The precipitation of plasmid DNA is due to specific complex formation hence the polycation concentration required was on the scale of 10 μg/ml. Contrary, when the DNA precipitation was due to the changes in solvent property, the required polymer concentration was about 100 mg/ml as in the case of PEG precipitation [21].

A clarified lysate was prepared and residual SDS was removed by the addition of zeolite. The plasmid precipitation with PDMDAAC was performed and after decanting the supernatant the pellet was re-dissolved in 2 M NaCl. The precipitation step resulted in 20-fold reduction in the volume of the plasmid solution. The analysis of the re-dissolved pellet by size-exclusion chromatography indicated that plasmid DNA was selectively precipitated while more than 95% RNA was removed. At least 90% of the proteins present in the initial lysate were removed as well. The plasmid recovery was 75–80%. Agarose gel electrophoresis verified that the purified plasmid was in supercoiled form (Fig. 8).

3.5. Removal of polycation by size-exclusion chromatography

We have shown the possibility to precipitate selectively plasmid DNA and to re-dissolve the formed pellet at high salt concentrations. If this precipitation concept is to be incorporated in a plasmid purification process, it is extremely important to show that the polycation can be removed after solubilization of the pellet. It was earlier shown for highly polymerised DNA, that no soluble polycation–DNA complexes were formed above salt concentrations 0.7–0.8 M NaCl [29]. It was thus reasonable to perform precipitation of plasmid DNA (pBluescript) and re-dissolve the pellet in 2 M NaCl, i.e. at much higher salt concentration than that sufficient for complete disruption of the complexes in the case of highly polymerised DNA. The re-dissolved pellet was then applied on a size-exclusion chromatography column in the presence of 2 M NaCl. The major fraction of the polycation was clearly separated from the plasmid, with a only a minor overlap in the tailing of the plasmid peak (data not shown).

4. Conclusions

Precipitation based on the formation of insoluble polyelectrolyte complexes is a powerful technique for capturing plasmid DNA from a clarified lysate. Selectivity for plasmid DNA is demonstrated and the precipitation is not, as in the case of precipitation by PEG or large amounts of salts, due to changes in the properties of the medium. Contaminants like RNA and proteins are left in the supernatant. By removing most of the major contaminant, i.e. RNA, following chromatography step will be much more effective since the capacity will be increased significantly. This straightforward technique results in high target recovery and removes a lot of contaminants at the same time as it significantly reduces the volume of the plasmid solution. The method utilises a non-expensive, commercially available polymer and only uses conventional unit operations like precipitation and dissolution, why straightforward scale-up is expected.

Acknowledgements

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