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Short communication

Clearance of host cell impurities from plasmid-containing lysates by boronate adsorption

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ABSTRACT

The ability of boronate adsorption to clear *Escherichia coli* impurities directly from plasmid-containing lysates (~pH 5.2) was evaluated. Results show that 3-aminophenyl boronate (PB) controlled pore glass (CPG) is able to adsorb not only those species that bear *cis*-diol groups (RNA, lipopolysaccharides-LPS), and are thus able to form covalent bonds with boronate, but also *cis*-diol-free proteins and genomic DNA (gDNA) fragments, while leaving most plasmid DNA in solution. Control runs performed with phenyl Sepharose and with PB-free CPG beads ruled out hydrophobic interactions with the phenyl ring and non-specific interactions with the glass matrix, respectively, as being responsible for RNA and gDNA adsorption. In batch mode, up to 97.6 ± 3.1% of RNA, 94.6 ± 0.8% of proteins and 96.7 ± 11.7% of gDNA were cleared after 30 min, with a plasmid yield of 64%. In fixed-bed mode, most of the plasmid was recovered in the flowthrough (96.2 ± 4.0%), even though the RNA (65.5 ± 2.8%), protein (84.4 ± 1.3%) and gDNA clearance (44.7 ± 14.1%) were not as effective. In both cases, the LPS content was removed to a residual value of less than 0.005 EU/ml. The method is fast and straightforward, circumvents the need for pre-treatment of the feed and may contribute to shorten plasmid purification processes, as the treated streams can proceed directly to the final polishing steps.

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1. Introduction

Gene therapy is an alternative for the prevention and treatment of genetic defects and acquired diseases [1]. Plasmid DNA (pDNA) vectors can be used in this context to deliver therapeutic transgenes into the target cells [2]. One of the keys for the development of such pDNA biopharmaceuticals is the availability of scalable production methods. After amplification in *Escherichia coli* (*E. coli*), pDNA must be separated from proteins (~55%, w/w), RNA (~20%, w/w), lipopolysaccharides (LPS, ~3%, w/w) and genomic DNA (gDNA, ~3%, w/w). The major challenges after alkaline lysis, the key disruption step in pDNA manufacturing, are the removal of RNA, LPS, and traces of gDNA, and the isolation of supercoiled pDNA isoforms from other less active variants [3,4]. Although several processes are available, new purification methods are actively pursued to facilitate the manufacturing of kilogram-amounts of pDNA with the stringent quality requirements stipulated by the FDA [5,6].

Affinity techniques use ligands that bind specifically and reversibly to the target molecules by intermolecular forces (e.g. ionic bonds, hydrogen bonds, van der Waals forces). Phenyl boronate (PB) ligands are able to form a pair of covalent bonds with molecules containing *cis*-diols via a reversible esterification reaction [7,8] (Fig. 1). In acidic solutions, boronic acids adopt a trigonal planar form which can revert to a tetrahedral boronate anion upon hydroxylation in alkaline conditions [9]. Both the acid and its conjugate base can bind to a diol compound. However, since the equilibrium constant for the tetrahedral form (K_{tet}) is usually higher than that of the trigonal form (K_{trig}), complexes are less stable in acidic conditions [7]. Most ligands used in boronate affinity adsorption are aromatic and thus also able to establish hydrophobic and aromatic π–π interactions. Secondary ionic interactions between boronates and diols are also possible through coulombic attraction or repulsion effects, hydrogen bonding via the hydroxyl groups and coordination interactions [9]. Boronate adsorption has been used to separate carbohydrates, nucleic acids and glycoproteins [9,10]. This work explores the ability of PB resins to adsorb RNA (via the 1,2-*cis*-diol in ribose) and LPS (via the *cis*-diols in the saccharide moiety) from plasmid-containing lysates. DNA is not expected to bind since deoxyribose lacks the 2′-hydroxyl group [9].

2. Materials and methods

2.1. Chemicals

Controlled porous glass (CPG) beads (74–125 μm, pore size ~1000 Å) with immobilized 3-aminophenyl boronic acid (ProSep®-

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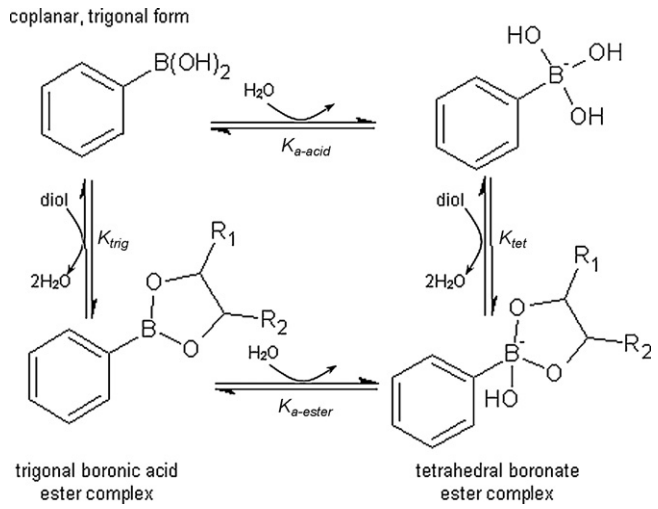

Fig. 1. Equilibria for boronic acid complexation with diols in aqueous solutions. Both the boronic acid and the boronate anion reversibly bind diols, forming a trigonal boronic acid ester (equilibrium constant K_{trig}) and a tetrahedral boronate ester (equilibrium constant K_{tet}), respectively.

PB) and with no derivatization were from Millipore (Bedford, MA, USA). All chemicals were of analytical grade and solutions were prepared in MilliQ water.

2.2. Plasmid DNA production

E. coli DH5α cells harbouring the 6050 bp pVAX1-LacZ plasmid (Invitrogen, Carlsbad, CA) were cultivated for 8 h in flasks with 250 ml of Luria Bertani medium and 30 μg/ml of kanamycin at 37 °C and 250 rpm. Cells were harvested with an OD600 nm ≈3.4 and alkaline lysis was performed as described earlier [11].

2.3. Phenyl boronate adsorption

PB adsorption was performed at room temperature in fixed-bed and batch modes using 3-aminophenyl boronate CPG beads. In batch experiments 560 μl of settled PB resin (equilibrated with water) and lysates (750 μl) were incubated in 1.5 ml microcentrifuge tubes for 1, 5 and 30 min. End-over-end mixing was performed during adsorption and elution. At each time point, the correspondent tube was centrifuged and the supernatant (~750 μl) was withdrawn. The material adsorbed was eluted in two consecutive steps with 750 μl of 1.5 M Tris (pH 8.7). After 1 h, beads were centrifuged, supernatants withdrawn and nucleic acids measured at 260 nm. Three independent experiments were performed and each time point was assayed in triplicate. For fixed-bed experiments, a column (5 mm i.d.) was packed with 746 μl of PB resin and connected to an Äkta purifier (GE Healthcare, Uppsala, Sweden). After equilibration with water (1.0 ml/min), 1 ml of lysate was injected, the column was washed with 10 ml of water and then eluted with 1.5 M Tris, pH 8.7. Fractions (0.5 ml) were pooled in two final samples (flowthrough and elution peaks), eluate absorbance was monitored at 260 nm. All runs were performed in triplicate, as three independent assays.

2.4. Analytics

Samples were analyzed by hydrophobic interaction HPLC [11,12]. A 15 PHE PE HIC column (4.6 mm × 10 cm) from GE Healthcare was equilibrated with 1.5 M ammonium sulfate in 10 mM Tris, pH 8.0 (1 ml/min). Lysate and samples from batch experiments were diluted 5-fold in this same buffer, whereas other samples were analyzed with no dilution. Following injection (30 μl), isocratic elution was performed with the equilibration buffer for 1.4 min and then with 10 mM Tris, pH 8.0 buffer for 0.9 min. At 2.3 min, the column was re-equilibrated with 1.5 M ammonium sulfate. Plasmid concentration was determined from a calibration curve (2.5–100 μg/ml). A HPLC purity was obtained by dividing the pDNA peak area by the total peak area in the chromatogram [11,12]. The amount of RNA removed was also estimated on the basis of the method.

Total protein in samples was quantified in triplicate using the BCA Protein kit from Pierce (Rockford, EUA). LPS concentration was assessed using the kinetic-QCL Limulus amoebocyte lysate (LAL) assay kit from Biowhittaker (Walkersville, USA). Genomic DNA in the feed lysate and in samples collected after batch and fixed-bed adsorption was estimated by real time PCR as described previously [13]. Samples were analyzed by agarose electrophoresis according to [12].

3. Results

Plasmid-containing alkaline lysates were contacted with PB matrix in batch and fixed-bed mode. The volumetric ratio of lysate to PB matrix was kept constant (~1.3) to facilitate comparison.

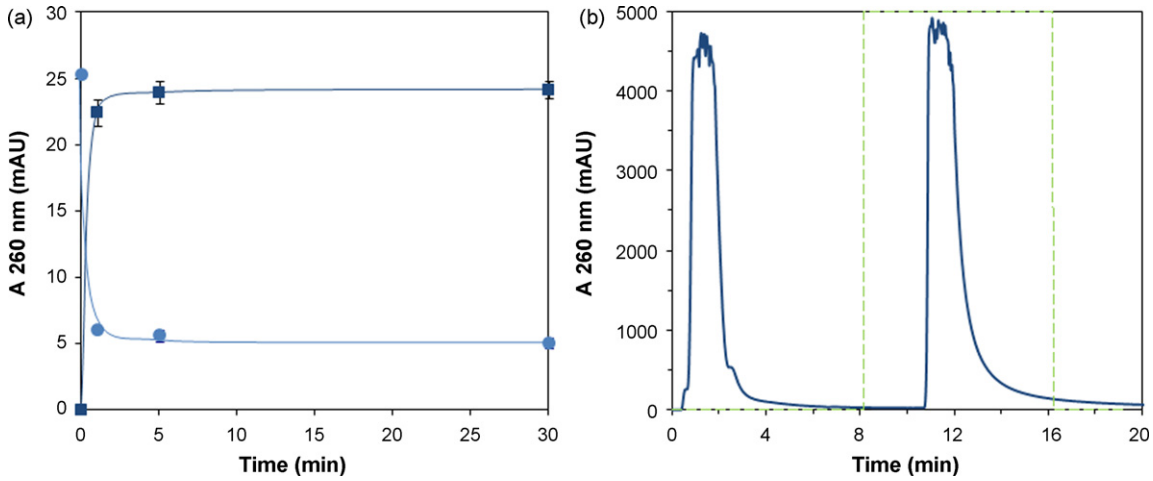

Fig. 2. Clearance of nucleic acids from plasmid-containing *E. coli* lysates by PB adsorption in batch (a) and fixed-bed mode (b). (a) The time course evolution of nucleic acids (measured by absorbance at 260 nm) in the liquid phase is shown over time of adsorption (●) and elution (■) for a 750 μl lysate sample. (b) The chromatogram shows the absorbance at 260 nm of the eluate stream after injection of 1 ml of lysate (solid line). The dashed line indicates the 1.5 M Tris step gradient.

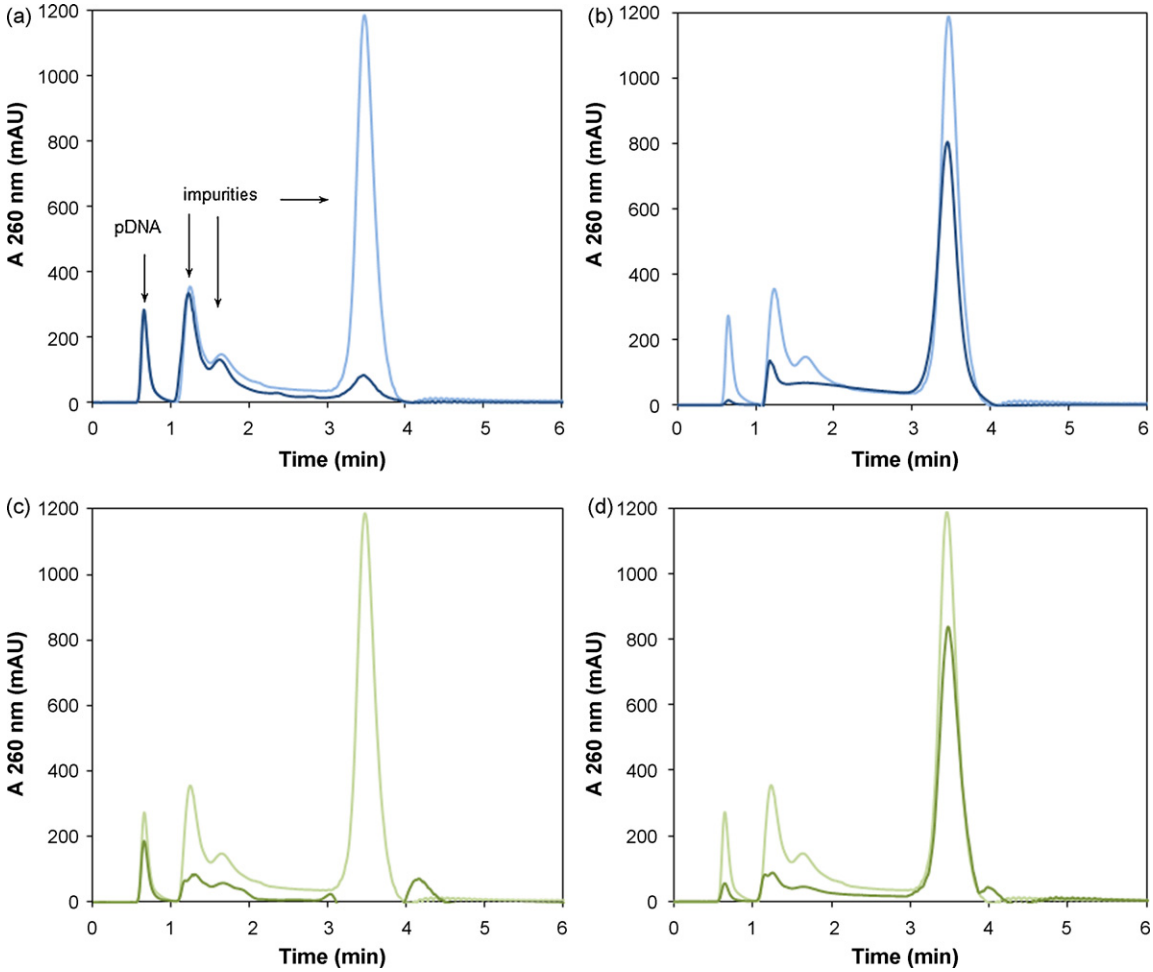

Fig. 3. HIC-HPLC analysis of PB adsorption in batch (a and b) and fixed-bed (c and d). In all panels, the chromatogram of the lysate feed (light line) is compared with the chromatograms (dark lines) of: (a) supernatant after a 5 min contact with the PB matrix, (b) material eluted from the matrix with 1.5 M Tris, (c) flowthrough pools and (d) elution pools.

Batch adsorption was monitored by recording the time course of the absorbance at 260 nm, A260 (Fig. 2a). After a sharp decrease in the first 5 min, the A260 stabilizes and remains unchanged for at least 30 min, indicating completion of adsorption. As expected, the elution of the bound material with 1.5 M Tris followed an opposite profile, with A260 increasing up to a maximum (Fig. 2a). The time course of fixed-bed adsorption was monitored by recording A260. The corresponding chromatogram shows an initial flowthrough peak of unbound material and a second peak of bound material after the onset of 1.5 M Tris elution (Fig. 2b). Although other eluents (e.g. 500 mM NaCl, water) were tested, only Tris was able to desorb bound species.

A further assessment of the PB adsorption was performed by HIC-HPLC analysis. The analytical chromatogram of the starting lysate (Fig. 3) displays a first peak of un-retained pDNA (0.7 min), followed by peaks of weakly retained low molecular weight RNA (*LMw* RNA, 1.0–2.5 min) and strongly retained high molecular weight RNA (*HMw* RNA, 3.0–4.0 min). The chromatograms of samples obtained during batch adsorption and elution show that a large fraction of *HMw* RNA is cleared after a 5 min contact with the PB matrix, whereas most pDNA and *LMw* RNA remain in the supernatant (Fig. 3a). This selectivity is confirmed by the analysis of the material eluted with 1.5 M Tris (Fig. 3b). The HPLC analysis of the flow-through (Fig. 3c) and elution (Fig. 3d) pools collected during fixed-bed adsorption produced qualitatively similar results, i.e. most pDNA and *LMw* RNA did not bind to the matrix and thus

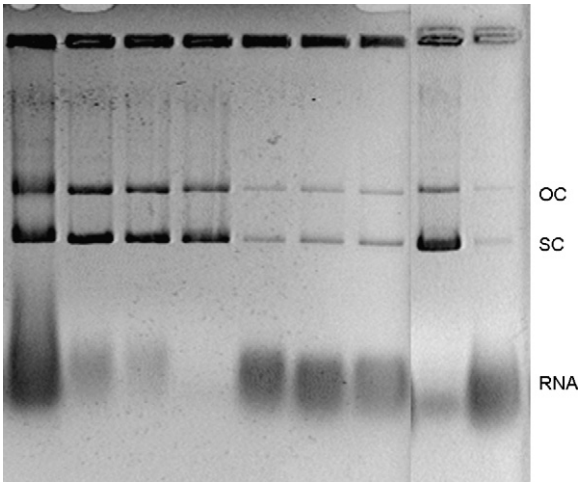

Fig. 4. Agarose gel electrophoresis analysis of batch and fixed-bed PB adsorption. Lane L: lysate, lane 1, 2 and 3: supernatant after 1, 5 and 30 min PB adsorption, respectively. Lane 4, 5 and 6: supernatant obtained after 1 hr elution (1.5 M Tris, pH 8.7) for 1, 5 and 30 min adsorption times, respectively. Lane 7 and 8: flowthrough and elution pools obtained during fixed-bed adsorption, respectively. The open circular (oc) and supercoiled (sc) plasmid isoforms are indicated.

Table 1
Clearance of host cell impurities by from plasmid-containing lysates by phenyl boronate adsorption. Plasmid yields, HPLC purity and RNA removal were determined by HIC-HPLC analysis. Protein, lipopolysaccharide and genomic DNA removal were estimated through BCA, LAL and RT-PCR assays, respectively.

Operation mode	Time (min)	Yield (%)	HPLC purity (%)	Purification factor	Impurity removal (%)			LPS (EU/ml)	Dilution factor
					RNA	protein	gDNA		
	1	70.5 ± 10.7	39.3 ± 12.0	7.2	96.9 ± 2.0	85.6 ± 1.8	53.3 ± 12.7	<0.005	0.73 ± 0.12
Batch[a]	5	65.1 ± 5.7	62.8 ± 26.8	11.4	98.4 ± 1.3	91.4 ± 1.0	72.0 ± 11.6	<0.005	0.67 ± 0.06
	30	64.0 ± 5.4	64.8 ± 24.9	11.8	97.6 ± 3.1	94.6 ± 0.8	96.7 ± 11.7	<0.005	0.66 ± 0.04
Fixed-bed[b]	–	96.2 ± 4.0	14.4 ± 0.75	2.6	65.5 ± 2.8	84.4 ± 1.3	44.7 ± 14.1	<0.005	0.50 ± 0.08

Lysate composition: [pDNA] = 55.2 μg/ml; [Protein] = 467.8 μg/ml; [gDNA] = 4146.7 μg/ml; [LPS] = 2007.7 EU/ml; HPLC purity = 5.5%.
[a] Results are the average of three independent experiments, each performed in triplicate.
[b] Results are the average of three independent experiments.

exited in the flowthrough, whereas *HMw* RNA bound strongly to the column and was only eluted by 1.5 M Tris.

Electrophoresis (Fig. 4) confirms that batch adsorption of RNA to the PB matrix is very fast and complete after 30 min (lanes 1–3). Furthermore, RNA readily desorbs from the matrix with 1.5 M Tris and a small fraction of pDNA binds to the resin (lanes 4–6). The gel analysis of the flowthrough (lane 7) and elution (lane 8) pools obtained during fixed-bed adsorption also confirmed the HPLC results in that a significant fraction of the RNA adsorbs, whereas most pDNA elutes in the flowthrough.

A quantitative analysis was performed on the basis of the HPLC results and of specific assays for the different *E. coli* impurities (Table 1). In batch mode up to 97.6 ± 3.1% of RNA, 94.6 ± 0.8% of proteins and 96.7 ± 11.7% of gDNA were cleared after 30 min. This corresponds to a 12-fold increase in the HPLC purity. A 36 ± 4.6% loss of pDNA to the adsorbent was also observed. In fixed-bed mode, most of the pDNA was recovered in the flowthrough (96.2 ± 4.0%), even though RNA clearance (65.5 ± 2.8%) was not as effective as in batch. Consequently, the HPLC purity increased only 2.6 fold. Protein and gDNA removal were about 84.4 ± 1.3 and 44.7 ± 14.1%, respectively. In both cases, the LPS content decreased from 2008 EU/ml in the lysate to a residual amount (<0.005 EU/ml).

4. Discussion and conclusions

The results show unequivocally that PB-CPG is able to adsorb not only those *E. coli* impurities which bear *cis*-diol groups (RNA, LPS), but also impurities that lack *cis*-diols like proteins and gDNA fragments, while leaving most pDNA in solution (Table 1). Furthermore, this selective separation was obtained by contacting lysates at pH ∼ 5.2 with the matrix. The extensive removal of RNA and LPS at such an acidic pH was somewhat surprising, since until very recently it was believed that boronate adsorption should be conducted at pH values above the pK_a of the ligands (8.9 for 3-aminophenyl boronic acid [8]). Additional experiments with feed samples prepared by precipitating lysate components with propan-2-ol, re-suspending them in water and adjusting pH to values >8.9, further showed that removal of RNA was always lower when compared with that obtained when contacting lysates at pH 5.2 with the matrix (not shown). This can be explained by the fact that RNA is degraded in alkaline conditions as a result of the hydrophilic attack of the 2′ OH group in ribose to the phosphodiester bond to form a cyclic phosphate. Hence, the resulting RNA fragments lack the *cis*-diol group and are unable to bind to PB-CPG. However, when the lysate components were precipitated with propan-2-ol and re-suspended in acidic solution (pH 5–7), the RNA adsorption yields were still lower when compared with those obtained when lysates were contacted with the PB-CPG. This indicates that some lysate components (e.g. potassium acetate) are important for RNA adsorption.

The fact that bound RNA and LPS were only desorbed when Tris, a competing *cis*-diol bearing species was added, supports the idea that covalent bonds are at the heart of the interaction. The possibility that hydrophobic interactions between the phenyl ring in the ligand and RNA could take place was discarded, since a control run with a phenyl Sepharose column showed that nearly all nucleic acids eluted in the flowthrough and that only small amounts of proteins bound to PB-CPG. Unlike RNA and LPS, the exact nature of the interactions between proteins and gDNA, and boronate ligands is more elusive, since these species are unable to esterify with boronic acid hydroxyl groups. To clarify this issue, lysate and gDNA samples were injected in a column packed with PB-free CPG beads. The hypothesis that non-specific interactions could take place with the glass matrix was ruled out since most nucleic acids eluted in the flowthrough, with only a small RNA fraction eluting with 1.5 M Tris (not shown). The binding of gDNA to the PB matrix could occur through the formation of charge transfer binary complexes between the vacant p orbital of the boron atoms and the phosphate groups in the DNA backbone [14]. However, the fact that pDNA does not bind seems to indicate that other interactions might be important. Genomic DNA is highly degraded in alkaline conditions becoming more hydrophobic and size variable and that might promote more hydrophobic interactions with the phenyl ring. As for proteins, charge transfer interactions might take place, together with hydrophobic interactions with the phenyl ring. The elucidation of the exact mechanisms through which RNA, gDNA, and proteins bind to PB is currently under investigation.

Although the ratio of feed to PB-CPG was kept constant, the contact time in fixed-bed was less than 1 min. This can explain the improved clearance of impurities obtained after 30 min of batch adsorption (Table 1). On the other hand, lower pDNA recoveries were obtained in batch mode. This is attributed to the fact that a significant portion of liquid remains trapped inside and between the settled beads. Since this was not accounted for, in batch experiments pDNA yield is underestimated, whereas impurity clearance is overestimated. In conclusion, boronate adsorption, both batch and fixed-bed modes, constitutes a simple, rapid and efficient step for the clearance of most impurities found in a typical *E. coli* lysate.

The key advantage of PB adsorption when compared with techniques like arginine, hydrophobic interaction and anion exchange chromatography is that it can be used very efficiently to process crude alkaline lysates directly, with no need for pre-purification or conditioning to adjust the ionic strength. On the other hand, the inability to concentrate plasmid is probably its most important shortcoming. Overall, we think that PB adsorption may be particularly valuable if combined with an additional step (e.g. ultrafiltration) to eliminate the remaining impurities and remove the excess of water.

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