Biporous Polymeric Microspheres Coupled with Mercaptopyridine for Rapid Chromatographic Purification of Plasmid DNA

Yuan Li, Xiao-Yan Dong, Yan Sun

Department of Biochemical Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, People's Republic of China

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ABSTRACT: A biporous absorbent coupled with mercaptopyridine was synthesized for the purification of plasmid DNA. Analyses by scanning electron microscopy and mercury intrusion porosimetry revealed that the matrix contained two families of pores, i.e., micropores and superpores. The superpores provided not only convective flow channels for the mobile phase, but also a large surface for biomacromolecules binding. So the chromatographic process can be operated at high flow rate with high column efficiency and low backpressure as identified on a 2-mL column. When 10 mL crude feedstock containing 3 mg of plasmid (5.4 kb pcDNA3) was loaded at a flow rate as high as 20 cm/min, the separation was finished in 10 min, and the plasmid was completely recovered with undetectable impurities of nucleic acids and proteins. The productivity was determined to be 9.0 g/L h, comparable to the pDNA productivity obtained using the commercial column. These results indicate that the biporous medium is promising for high-throughput purification of plasmid DNA.
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Key words: macroporous polymers; adsorption; suspension polymerization; plasmid; purification

INTRODUCTION

Owing to the safety concerns associated with viral vectors, there is a growing interest in the use of nonviral vectors such as plasmid DNA in gene therapy applications and vaccine. To produce large quantities of plasmid DNA, an efficient large-scale purification process that can meet specifications in purity, needs to be developed. Today, there are a lot of different approaches to purify plasmid DNA. Some of them are based on the precipitation of RNA and proteins in a DNA concentrate,[1] while most are concerned with chromatographic separations.[2–8] In general, liquid chromatography is the most important tool for the analytical and preparative separations of plasmid DNA. However, chromatographic matrices used to purify plasmid from cell lysate are almost porous matrices composed of agarose, dextran or cellulose and the severe internal mass transfer resistance and the compressibility of the adsorbents limits their application to high speed operation. Moreover, the chromatographic matrices are originally designed for protein chromatography. Since plasmid DNA is much larger than proteins, most protein-directed chromatographic media do not give favorable results for plasmid purification. That is, DNA molecules are unable to utilize the binding sites that are located in the internal pores and the binding is largely a surface adsorption phenomenon.[6–9] Therefore, the capacities of traditional packings for plasmid DNA are much lower than those reported for proteins.[10,11]

To overcome these problems, flow-through chromatography is considered as a favorable alternative. The packing materials for flow-through chromatography have large through-pores or superpores.[12] These wide pores made it possible for the mobile phase to flow through the pores convectively, so the interior mass transfer is greatly enhanced and the packing shows high efficiency and dynamic capacity for biomacromolecules at high mobile-phase flow rates. This provides more opportunity for high-speed chromatographic purification of proteins and nucleic acids at high loading capacities.[5]

In previous publications, the authors have reported a biporous medium for high-speed chromatography separation of proteins by ion exchange chromatography[13] and plasmid DNA by hydrophobic interaction chromatogrpahy.[14] In this work, we coupled 2-mercaptopyridine to the biporous matrix to produce a biporous thiophilic medium for plasmid purification. It has been reported that mercaptopyridine ligand showed favorable performance in plasmid DNA purification

Correspondence to: Y. Sun (ysun@tju.edu.cn).
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modulated by high concentrations of lyotropic salt.[15] Because the mercaptopyridine ligand binds proteins and RNA preferentially, we have performed the purification by the flow-through mode of pDNA. Because the molecular masses of proteins and RNA are much smaller than pDNA, higher binding capacities of the adsorbent for these relatively low molecular-mass substances would be possible. This would therefore lead to the partial solution to the problem of the low pDNA capacity of common porous media. Consequently, high-capacity purification of pDNA by using the mercaptopyridine-ligand medium is expected because the pDNA could flow through the column without binding. Here we examined the chromatographic behavior by coupling the ligand to the customized biporous resin. Our aim is to realize a high flow-rate chromatographic operation with the biporous adsorbent, thus leading to a high throughput production of plasmid DNA.

EXPERIMENTAL

Materials

Ethylene dimethacrylate (EDMA) was obtained from Sigma (St. Louis, MO). Yeast extract and tryptone were from Oxoid (Hampshire, England). The DNA marker DL15000 was from TaKaRa Biotech (Dalian, China). Glycidyl methacrylate (GMA) (99%) was purchased from Suzhou Anli Chemical Company (Jiangsu, China). Agarose powder, ampicillin, tris(hydroximethyl) methylamine (Tris), sodium salt of ethylenediaminetetraacetic acid (EDTA), sodium dodecylsulfate (SDS) and other materials were all received from Dingguo Biotech (Beijing, China).

Fermentation and crude pcDNA3 preparation

Escherichia coli DH5α strain harboring the plasmid pcDNA3 of 5.4 kb was kindly provided by the Hematology Institute of Chinese Academy of Medical Sciences (Tianjin, China). The strain was stored in LB medium plus 20% glycerol at −80°C. A transformed strain was grown in 2×YT medium containing 150 μg/mL ampicillin at 37°C by shaking culture in 2-L flasks at 200 rpm for 14–16 h till an optical density of ~ 4 was reached. The cells were harvested by centrifugation at 5000 g for 15 min at 4°C.

Crude pcDNA3 was prepared by alkaline lysis and isopropanol precipitation based on the method described in literature.[16] The precipitate was dissolved in TE buffer (25 m*M* Tris-HCl, 10 m*M* EDTA, pH 8.0). Next, solid ammonium sulfate was dissolved in the plasmid solution up to a concentration of 2.5*M*, followed by 15-min incubation on ice. The precipitated proteins and RNA were removed by centrifugation at 10,000 × *g* for 15 min at 4°C. The supernatant was used as the feedstock for further plasmid DNA purification by chromatography.

Plasmid standard

Plasmid standard of high purity was prepared using a plasmid purification kit from Promega Corp. (Madison) according to the instructions of the manufacturer.

Preparation of chromatographic adsorbent

The biporous resin (BiPR) was synthesized by the radical suspension–polymerization with a mixture of cyclohexanol and dodecanol as the liquid porogenic agent and calcium carbonate granules as the solid porogenic agent.[13,14] Composition of the polymerization mixture for preparing the beads was 24/16/45/5/10(GMA/EDMA/cyclohexanol/dodecanol/solid granule in volumetric ratio). The content of free radical initiator AIBN was 1% w/v (with respect to monomers). The beads prepared were thoroughly washed with a large amount of hot deionized water (50–60°C). The solvent porogenic agents, cyclohexanol and dodecanol, were removed by extraction with ethanol under reflux for 24 h in a Soxhlet extraction apparatus, while the solid porogenic agent, calcium carbonate, was removed by slowly adding of glycine-HCl buffer (100 m*M*, pH 3.0). When no bubbles of CO_2 could be detected and the pH value of the glycine-HCl buffer did not change, the biporous resin was filtered and rinsed with water until a neutral pH was reached. Then the biporous resin was dried in vacuum at 50°C overnight.

Then, the medium was modified by coupling mercaptopyridine. In the procedure, 5.0 g of BiPR, 11.2 g of 2-mercaptopyridine and 0.5 g of sodium hydroxide were added to 50 mL of dioxane, and the mixture was stirred and heated to 60°C and kept at this temperature for 6.5 h. Thereafter, the biporous resin was thoroughly washed with dioxane, ethanol, and an excess of distilled water. The epoxide groups remaining on the polymer were reduced to hydroxyl groups by suspending the beads in 100 mL of 0.1*M* $NaBH_4$ solution and shaking the suspension overnight in a shaking incubator of 140 rpm at room temperature. The BiPR modified with 2-mercaptopyridine (MP-BiPR) was thoroughly washed with an excess of ethanol and distilled water. The 2-mercaptopyridine group density on the MP-BiPR was determined to be 0.41 mmol/g matrix by elemental analysis (sulfur) (Elemental Vario EL, Germany).

Characterization of the matrices

The pore structure of the beads was observed by scanning electron microscopy (SEM) on an XL30 ESEM scanning microscope (Phillips, The Netherlands). The

pore size distribution was determined by mercury porosimetry using a Quantachrome Poremaster-60 mercury porosimeter (Quantachrome Corp.). Particle size distribution of the matrix was measured with Mastersizer 2000 particle size analyzer (Malvern Instrument, UK).

Analyses

Plasmid and other nucleic acids were analyzed by electrophoresis using 0.7% agarose gel in 40 m*M* Tris base, 20 m*M* sodium acetate, 2 m*M* EDTA (pH 8.3). The electrophoretic separation was performed at a constant voltage at room temperature for 1 h. Nucleic acids were stained with 1 μg/mL of ethidium bromide solution included in the gel.

Plasmid concentration and plasmid purity in the pcDNA3 sample was measured by size exclusion HPLC (SE-HPLC) on the Agilent 1100 system (Agilent Technologies, DE). Injection of 20 μL of sample was followed by isocratic elution with buffer (25 m*M* Tris, 0.3*M* NaCl and 1 m*M* EDTA, pH 7.5) for 70 min at 0.4 mL/min on TSKgel G-DNA-PW followed by TSKgel G-6000-PW_{XL} column (Tosoh Corp., Japan) in series. To prepare a calibration curve, plasmid standards of concentrations ranging from 5 to 200 μg/mL were injected to the column and the area under the peak of absorbance at 260 nm was plotted against the plasmid concentration. Samples of unknown plasmid concentration were then analyzed by the SE-HPLC, and the concentration was determined from the calibration curve. The percentage of plasmid peak area in each chromatogram was thus used as a measure of the sample purity.[17]

The concentration of nucleic acids in the crude plasmid preparation was also measured by Genequant DNA/RNA calculator (Amersham Biosciences, Uppsala, Sweden). Together with the results of SE-HPLC for the plasmid analysis, RNA concentration in the crude feedstock was determined.

Protein concentration was determined by Micro BCA protein assay kit from Pierce (Rockford) according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as the standard in the range of 0.5–20 μg/mL.

Plasmid purification by chromatography

Chromatographic separation was performed on the äKTA FPLC system (Amersham Biosciences, Uppsala, Sweden). An HR 5/10 column (5 mm I.D., 10 cm in length) from Amersham Biosciences packed with MP-BiPR particles was connected to the chromatograph. The outlet stream was continuously monitored by both a conductometer and a UV monitor at 254 nm. All chromatographic experiments were performed at room temperature. In the purification experiments, the crude pcDNA3 preparation obtained after precipitation with $(NH_4)_2SO_4$ was used as the feedstock. Prior to a purification experiment, the column was equilibrated with 10 column volumes (CVs) of buffer A (50 m*M* Tri-HCl, 1*M* $(NH_4)_2SO_4$, 10 m*M* EDTA, pH 7.2). To optimize the separation, injection of samples was performed using a 100 μL loop. After elution of unbound or weakly retained species with buffer A, the ionic strength (ammonium sulfate concentration) of the buffer was linearly decreased by the change of buffer A to buffer B (50 m*M* Tri-HCl, 10 m*M* EDTA, pH 7.2).

The mercaptopyridine ligand binds proteins and RNA more strongly than plasmid DNA.[15] Thus, the plasmid would be recovered as a flow-through fraction before the breakthrough of other impurities (proteins and RNA). To detect the breakthrough volume of the impurities, 20 mL of the feedstock was loaded to the column through the Superloop 50 mL (Amersham Biosciences) at 1 mL/min. The effluent was collected every 1 min and analyzed by SE-HPLC. By the breakthrough volume of the impurities, a proper loading volume of the feedstock was determined for high-throughput purification at high flow rate, 4 mL/min (20 cm/min).

After each run, the column was cleaned with 15 CVs of 0.5*M* of sodium hydroxide solution. Then, the column was equilibrated with buffer A. When the UV signal reached the baseline, the next sample was loaded.

RESULTS AND DISCUSSION

Properties of the biporous matrix

The volume-weighted mean diameter of the matrix particles was determined to be 41 μm and 80% by volume of the particles were in the range of 29–54 μm. Figure 1 shows an SEM photograph of the matrix. It can be clearly seen from the picture that the matrix is full of micron-sized wide pores. In addition to the SEM observation, the pore size distribution of the matrix was

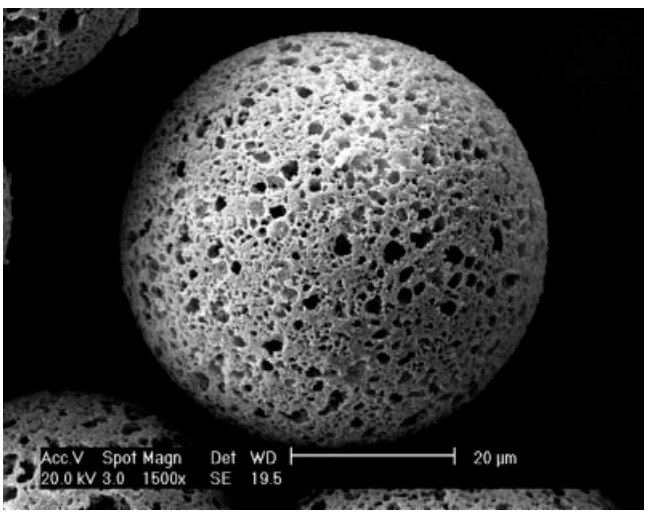

Figure 1 A scanning electron micrograph of the biporous microsphere.

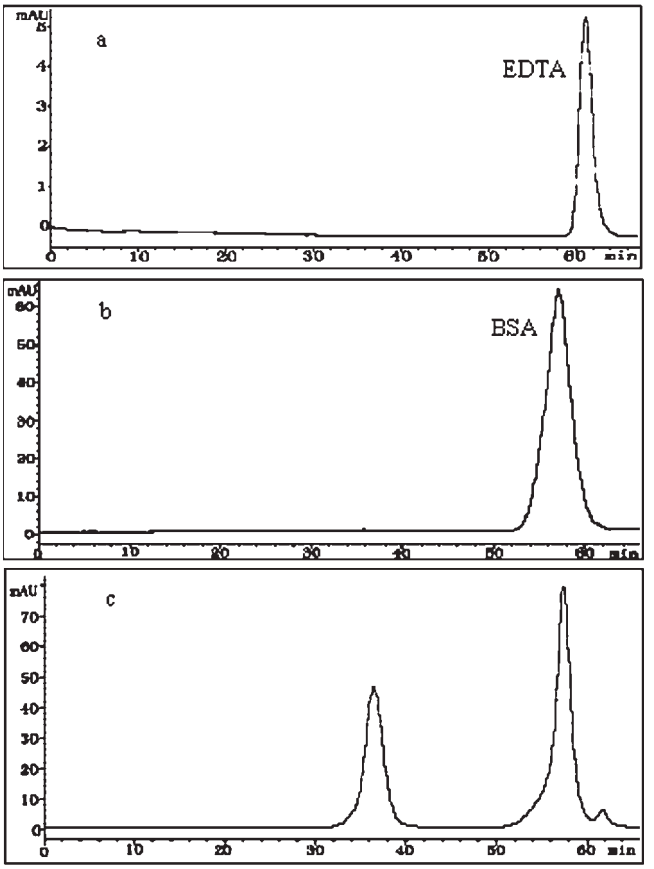

Figure 2 SE-HPLC analyses of (a) EDTA, (b) BSA and (c) crude plasmid preparation. The sample concentrations of EDTA, BSA and crude plasmid solution were 10 m*M*, 5 and 0.3 mg/mL, respectively.

measured by mercury intrusion porosimetry. The results indicate that the micropores of 2–100 nm took 31% volume of the total pores while the superpores of 0.5–7.3 μm were about 23%. The other pores were in the range of 100–500 nm.

The structure of the biporous resin made it possible for the mobile phase to flow convectively through the superpores.[13] It was found that the backpressure of the column increased linearly with flow velocity up to 35 cm/min and the backpressure was still below 1.5 MPa at the velocity as high as 35 cm/min, which was much lower than the column packed with the microporous beads (2.7 MPa, the microporous bead was prepared by the same method without the use of calcium carbonate as the solid porogenic agent.[13]) In addition, the superpores in the biporous resin are expected to provide a large surface area for the binding of proteins and high-molecular-mass nucleic acids during the convective flow of mobile phase through them.

Separation of pcDNA3 by chromatography

The feedstock obtained by ammonium sulfate precipitation contained 0.30 mg/mL of pcDNA3, 0.44 mg /mL of RNA and 54 μg/mL of protein. Before the separation of pcDNA3, analytical experiments were carried out to identify the chromatographic peaks of plasmid preparation in the SE-HPLC. Figure 2(a, b) shows the chromatograms of EDTA and BSA solution, respectively. From these figures, we know that the EDTA was eluted at about 61 min while BSA was done at 58.5 min. Figure 2(c) shows the SE-HPLC analysis of the crude plasmid preparation. There were three peaks in this profile. Eon-Duval and Burke[6] have shown that plasmid DNA (5.9 kb) and RNA can be completely separated by the SE-HPLC; the first peak was plasmid while RNA was eluted from the SE-HPLC columns at approximately the same volume as that of the second peak in Figure 2(c). So, in the crude preparation, RNA and the proteins were eluted as an overlapped peak [second peak in Fig. 2(c)] and the third small peak was low-molecular-mass substances such as EDTA because its retention time was the same as that in Figure 2(a).

To examine the binding behavior of the plasmid, RNA and proteins to MP-BiPR, chromatographic separation of the plasmid preparation was performed at different salt concentrations. Figure 3 is the chromatogram of plasmid preparation on the MP-BiPR column with buffer B (no ammonium sulfate) as a loading buffer and eluant. It can be seen that two peaks were obtained at the condition of low salt concentration. The two peaks in the Figure 3 were collected and analyzed by the SE-HPLC (Fig. 4). As can be seen, peak 1 in Figure 3 was the pcDNA3, while peak 2 was composed of the low-molecular-mass RNA and proteins. Therefore, at the low salt concentration, plasmid, RNA and protein were

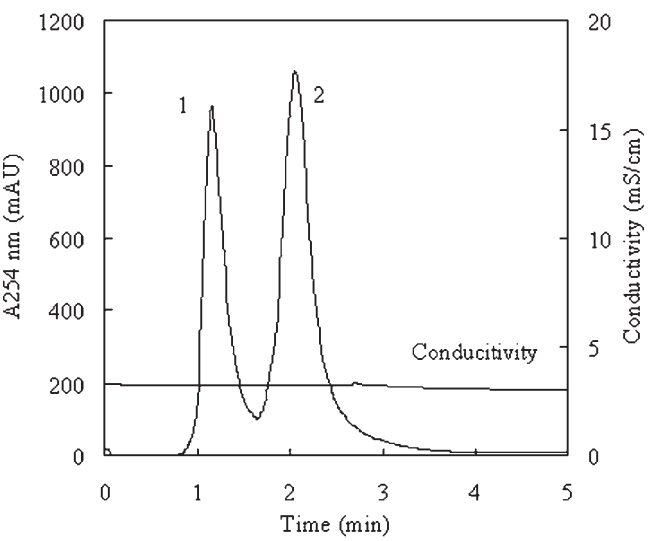

Figure 3 Chromatographic profile of the plasmid preparation on the column packed with MP-BiPR. The feedstock in buffer B (0.1 mL) was loaded and the column was developed with buffer B at 1 mL/min.

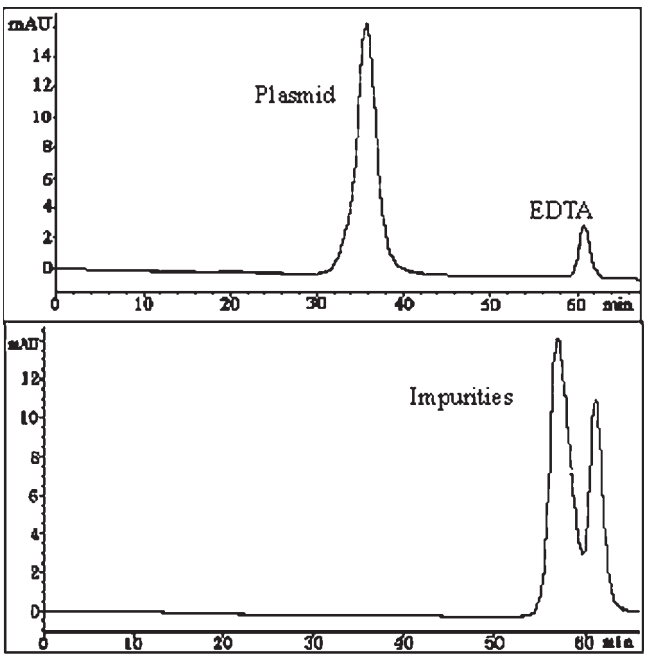

Figure 4 SE-HPLC analyses of the fractions collected from the chromatography shown in Figure 3. The top panel shows the SE-HPLC profiles of peak 1 in Figure 3 and the bottom shows those of peak 2 in Figure 3.

unbounded or weakly bounded to the MP-BiPR and plasmid can be separated from RNA and proteins by size exclusion mechanism.[17]

Figure 5 is a chromatographic profile of the plasmid preparation on the MP-BiPR column with buffer A as a loading buffer (containing 1*M* ammonium sulfate). After elution of unbound and weakly retained species with buffer A, ammonium sulfate concentration in the mobile phase was linearly decreased by the change of buffer A to buffer B in 2 min. The two peaks were collected and analyzed by SE-HPLC. As shown in Figure 6, the unbound fraction (peak 1 in Fig. 5) was pure plasmid, while the bound species (peak 2 in Fig. 5) were RNA and proteins. From the results shown in Figures 5 and 6, we know that the plasmid was little retained by the column while RNA and proteins were done in buffer A. Thus, the plasmid can be separated from RNA and proteins as a flow-through fraction when it is loaded in buffer A.

To test the effect of mercaptopyridine ligand on the retention behavior of the components in the plasmid preparation, we have also performed a control experiment with a column packed with unmodified BiPR. The result revealed that in buffer A all components in the plasmid preparation were little bound by BiPR, and they were eluted with buffer A within 5 min. Therefore, the retention of the impurities by MP-BiPR as shown in Figure 5 was confirmed to be due to the coupled 2-mercaptopyridine ligand.

Lemmens et al.[15] have found that supercoiled plasmid DNA could be isolated from other isoforms of plasmid DNA by Sepharose 6FF coupled with 2-mercaptopyridine in the presence of ammonium sulfate. However, this was not realized by the present column when adjusting the concentration of ammonium sulfate in the loading buffer. Thus, we emphasized our separation work on high-throughput production of plasmid DNA by recovering it as a flow-through fraction.

Frontal analysis and high-throughput purification

To recover the plasmid DNA as a flow-through fraction before the breakthrough of RNA and proteins, the breakthrough volume of RNA and proteins needs to be determined first. Thus, a frontal analysis experiment was performed by loading 20 mL the crude plasmid preparation at 1 mL/min and the effluent pools were analyzed by SE-HPLC, agarose electrophoresis and BCA protein assay at different time intervals (data not shown). As a result, it was observed that the plasmid flowed through the column as a pure component in the first 14-mL loading. Thereafter, RNA began to appear in the effluent stream. This implies that at the chromatographic condition, up to 14 mL of the crude feedstock could be applied to the 2-mL column with little breakthrough of the impurities. As the concentration of RNA in the feedstock was 0.44 mg/mL, thus, the dynamic capacity of the column for RNA is calculated to be 3.1 mg/mL column at the flow rate of 1 mL/min (5 cm/min). It is considered that the superpores provided more binding sites for RNA.

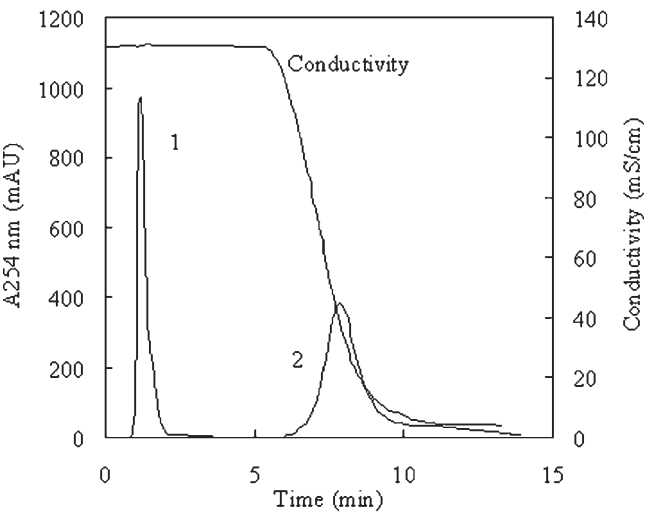

Figure 5 Chromatography of the plasmid preparation on the column packed with MP-BiPR at 1 mL/min. The feedstock in buffer A (0.1 mL) was applied and the column was washed with buffer A for 5 min. Then the linear gradient of 0–100% buffer B was accomplished in 2 min.

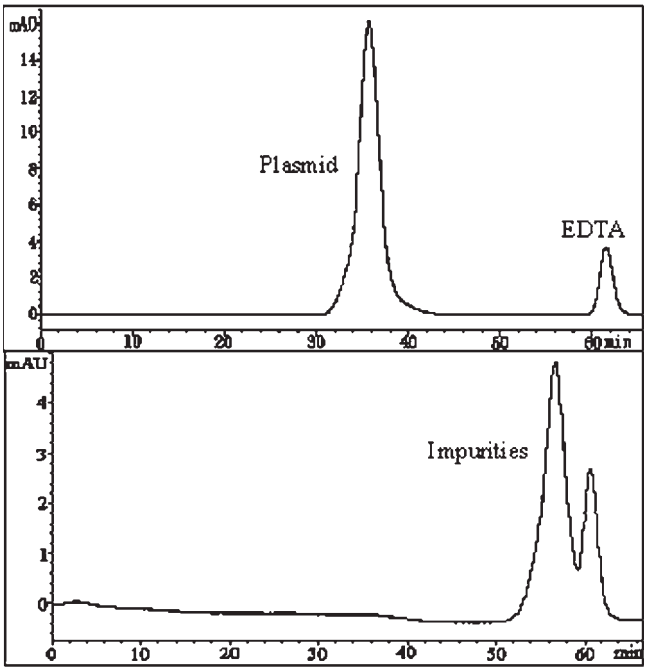

Figure 6 SE-HPLC analyses of the fractions collected from the chromatography shown in Figure 5. The top shows the SE-HPLC profiles of peak 1 in Figure 5 and the bottom shows those of peak 2 in Figure 5.

The dynamic capacity of the column for bovine serum albumin has been determined to be 35 mg/mL column, and decreased only slightly up to a flow rate of 20 cm/min.[13] Hence, we performed pcDNA3 purification by loading 10 mL of the feedstock at an elevated flow rate of 4 mL/min (20 cm/min), which was four times higher than that in the frontal analysis experiment. As given in Figure 7, at the elevated flow rate and high feedstock loading, the chromatographic profile was approximately the same as that in Figure 5. The two wide peaks in the chromatogram were collected and analyzed by SE-HPLC (Fig. 8) and agarose electrophoresis (Fig. 9). It can be seen from Figures 8 and 9 that the unbound fraction (peak 1 in Fig. 7) did not contain RNA, while plasmid did not appear in the bounded species (peak 2 in Fig. 7). Furthermore, protein was not detected by BCA protein assay in the plasmid pool. From the results, we can conclude that the plasmid DNA was purified with complete recovery, and the main impurities, RNA and proteins, in plasmid preparation, were removed completely with the one step chromatographic run. Moreover, the purification was carried out at a flow velocity of 20 cm/min, which is a superficial velocity 5–10 times higher than normal preparative chromatography.[6,18] At this high flow rate, 3.0 mg plasmid was purified within 10 min by the 2-mL MP-BiPR column. The productivity of the separation process was thus estimated at 9.0 g/L h (= 3.0 mg/2 mL/10 min), comparable to the pDNA productivity obtained using the commercial CIM (Convective Interaction Media) radial flow monoliths in an anion exchange chromatographic mode (8.7 g/L h).[19]

The MP-BiPR column was used for more than 10 runs from the optimization experiments till the high loading purification. After each run, the column was regenerated with 0.5*M* of NaOH solution. During the process, little change of the column backpressure and

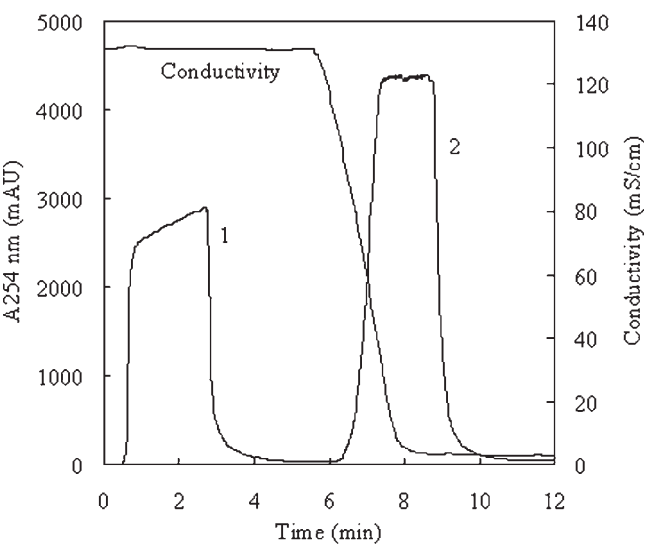

Figure 7 Purification of pcDNA3 by loading 10 mL of the crude plasmid preparation. The chromatography was operated at 4 mL/min (20 cm/min). The column was washed with buffer A for 5 CVs, then the linear gradient of 0–100% buffer B was accomplished in 2 min.

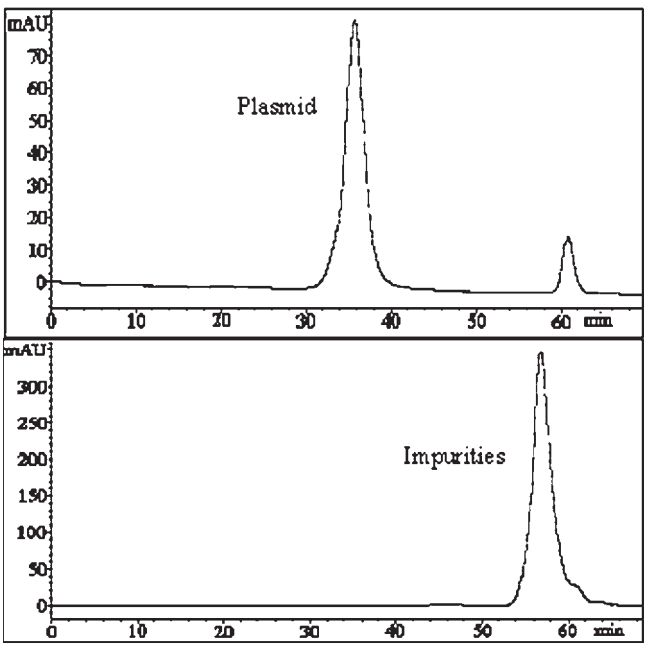

Figure 8 SE-HPLC analyses of the fractions collected from the chromatography shown in Figure 7. The top shows SE-HPLC profiles of peak 1 in Figure 7 and the bottom shows those of peak 2 in Figure 7.

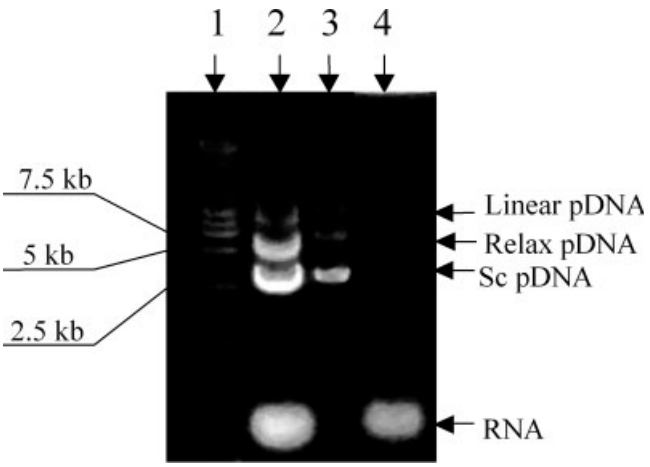

Figure 9 Electrophoresis of fractions collected from the chromatography shown in Figure 7. Lane 1, DNA marker; lane 2, crude pcDNA3 preparation; lane 3, peak 1 in Figure 7; lane 4, peak 2 in Figure 7.

separation capacity was observed. This demonstrated the stability and reproducibility of the biporous adsorbent based on the crosslinked GMA and EDMA. Because of the stability of the GMA-EDMA matrix, it has been developed to commercial monolith columns.[19] All the results indicate that the customized biporous adsorbent is promising for high-throughput purification of plasmid DNA.

CONCLUSIONS

In the present article, a biporous medium coupled with 2-mercaptopyridine has been prepared for the purification of plasmid DNA. At a high salt concentration such as 1*M* ammonium sulfate, the MP-BiPR column adsorbed RNA and proteins, while the plasmid DNA flowed through the column without binding. The biporous feature of the medium made it possible for the mobile phase to flow convectively through the superpores inside the particles, so the intraparticle mass transport was enhanced and chromatography could be carried out at high flow rate up to 20 cm/min. Furthermore, the superpores provide more binding sites for the macromolecules such as RNA during the convective flow of mobile phase through them, giving a dynamic capacity of 3.1 mg/mL for RNA. When 10 mL of the crude plasmid preparation containing 3 mg pcDNA3 was loaded on a 2-mL column packed with the MP-BiPR at a flow velocity of 20 cm/min, the plasmid DNA was purified with complete recovery as a flow-through fraction, and the main impurities, RNA and protein in plasmid preparation, were removed completely with the one step chromatographic run. It is notable that the flow velocity is 5–10 times higher than normal preparative chromatography. These results indicate that the customized biporous medium is promising for high-speed purification of plasmid. If more RNA and proteins were removed in the primary processing steps, the plasmid purification capacity of the column would be further increased.

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