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Single step purification of plasmid DNA using peptide ligand affinity chromatography

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ABSTRACT

Single step affinity chromatography was employed for the purification of plasmid DNA (pDNA), thus eliminating several steps compared with current commercial purification methods for pDNA. Significant reduction in pDNA production time and cost was obtained. This chromatographic operation employed a peptide–monolith construct to isolate pDNA from *Escherichia coli* (*E. coli*) impurities present in a clarified lysate feedstock. Mild conditions were applied to avoid any degradation of pDNA. The effect of some important parameters on pDNA yield was also evaluated with the aim of optimising the affinity purification of pDNA. The results demonstrate that 81% of pDNA was recovered and contaminating gDNA, RNA and protein were removed below detectable levels.

1. Introduction

Plasmid DNA (pDNA) is a promising vector for gene therapy and DNA vaccination, particularly with regards to safety in terms of eliminating oncogene activation and unintended immunological reactions [1–6]. For such clinical applications, there is a growing demand for production of large quantity. pDNA is usually produced by fermentation and purification employing a sequence of unit operations [7,8], for example, ion exchange chromatography [9,10], size exclusion chromatography [11,12], hydrophobic interaction chromatography [13,14] and reverse phase chromatography [15]. These purifications exploit the physicochemical properties of nucleic acids in non-specific capture systems. However, the non-specific nature of these methods has been associated with the problem of co-purification of impurities, i.e. RNA, genomic DNA and endotoxins [7,8,16,17].

Current processes of purification of pDNA require several chromatographic steps [8,16,18], which make pDNA production not only time-consuming but also costly [1]. However, affinity chromatography may eliminate some purification steps as illustrated in Fig. 1. Affinity chromatography plays a powerful role in separation technology as this technique enables purification of a biomolecule on the basis of interaction between the target plasmid and ligand. High selectivity makes this technique capable of achieving purification in a single process [19]. Affinity purification mechanisms employ a stationary immobilized ligand attached to an insoluble solid phase matrix. A mobile liquid phase containing the target biomolecule is applied to the matrix under conditions that favour its specific binding to the immobilized ligand. Unbound and weakly bound substances are washed away and the substance of interest can be recovered by changing the process conditions to those which favour its desorption. Affinity chromatography has the purification power to eliminate steps, increase yields and downsize capital equipment, and thereby improve process economics [20].

Development of affinity chromatography can be difficult if the elution of the product cannot be achieved without extreme conditions. Previous affinity ligands include a sequence-specific zinc-finger protein [21] and triplex DNA formation [22,23], which are associated with the problem of ligand contamination and slow binding kinetics, respectively. The interaction between lac repressor protein and lac operator sequences, or lacI–lacO interaction, was applied for purification of short DNA sequences containing the lacO [24], and for the enrichment of viral vectors and the sequence-specific recovery of DNA from transgenic mice [25]. High selectivity of lacI–lacO interaction makes it suitable for pDNA purification. Darby and Hine (2007) have shown that pDNA can be purified using a lacI fusion protein as an affinity ligand, but the protein is expensive [26,27]. Different stationary phases employing LacI-His6 affinity ligand for prepurified pDNA have been evaluated [28]. Hasche and Voß [28] found that the interaction between repressor molecules and RNA was not detectable when the interaction involved double stranded DNA in the form of a short operator sequence and pDNA. They recommended that reducing the size of the ligands by only

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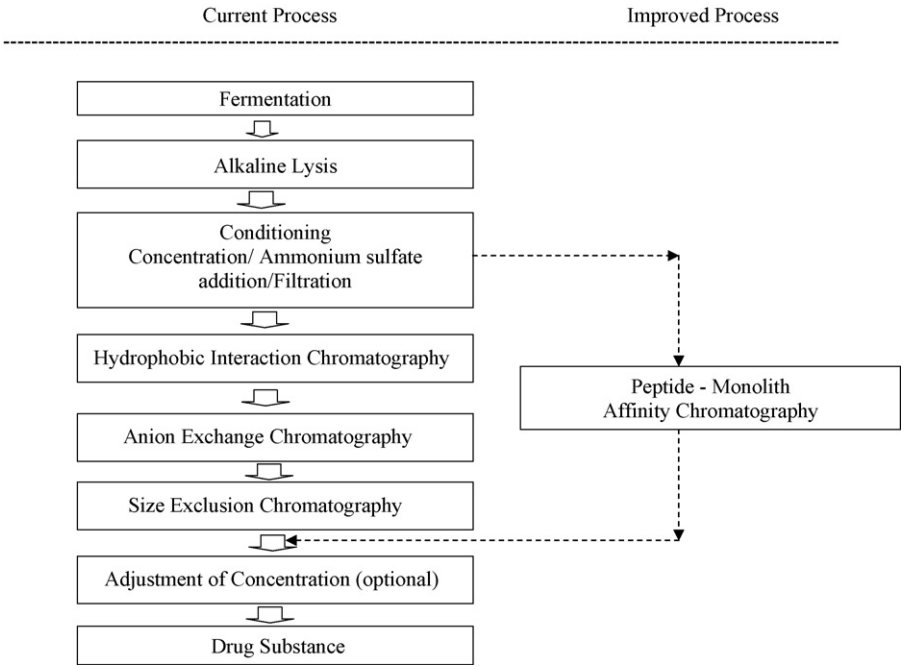

Fig. 1. A current commercial process flow sheet for purification of plasmid DNA. pDNA is purified by a series of three chromatographic steps, in addition to several concentration steps. The improved manufacturing process will replace the three chromatographic steps with a peptide ligand affinity step (16mer peptide–monolith). The new step in improved process is shown by dash line.

containing the DNA binding domain could increase DNA binding capacity of immobilized proteins. Forde et al. (2006) found that a 64mer peptide representing a full DNA binding domain region of lacI had very high-binding affinity (dissociation constants in the nanomolar range) that was difficult to disrupt without extreme pH [29].

An ideal ligand needs to have appropriate binding kinetics so that the pDNA can be effectively captured and eluted. To achieve these conditions, a 16mer peptide representing helixII of DNA binding domain of lac repressor has been selected to use as a ligand in this study based on its binding kinetics to pDNA [30]. A reliable affinity purification requires not only optimized ligands but also a suitable stationary matrix. If the pore size of the matrix is too small, pDNA binding will be restricted due to non-accessibility of the ligands in the matrix. A monolith is known for fast separation with its large pore diameter, which makes large molecule pDNA easily access with limited mass transfer resistance [31–33]. In this study, a monolithic matrix was synthesized for pDNA purification purposes. To our knowledge, this is the first time to utilize a peptide ligand immobilized on a customized monolith to purify pDNA. The objective of this study is to evaluate whether the interaction between pDNA and 16mer peptide is sufficient to capture pDNA in a chromatographic system, the effects of NaCl concentration, flow rate and residence time on pDNA purification were also investigated.

2. Materials and methods

1 kb DNA ladder (BioLabs Inc., New England). Agarose and PureYield™ Maxiprep DNA Purification System were purchased from Promega (Madison, WI, USA). *Escherichia coli*, DH5α (EndA⁻) and pUC19 plasmid (0.01 μg/l) were purchased from Invitrogen (Victoria, Australia). Ethidium bromide, ethylenediaminetetraacetic acid (EDTA, Serva, USA). Sodium chloride and Tris (hydroxymethyl) aminomethane were purchased from Amresco (Ohio, USA). Sodium hydroxide, Tris–HCl, Picogreen assay and other chemicals were purchased from Sigma–Aldrich (St. Louis, USA) and of analytical grade if not stated otherwise.

The Duoflow biological chromatographic unit and gel electrophoresis systems were purchased from Biorad Laboratories Inc., CA, USA. The spectrophotometer (Shimadzu UV-2450) was from Japan.

2.1. Bacterial production and isolation of plasmid DNA

The plasmid pUC19 is 2686 bp in size and contains two lacO-binding domains: $lacO_1$ from bases 180 to 196 and $lacO_3$ from bases 88 to 104. PUC19 (0.01 μg/l, Invitrogen) was transformed and propagated in *E. coli* DH5α (EndA⁻, Invitrogen, Australia). A culture of *E. coli* DH5α was grown in LB medium containing 100 μg/ml ampicillin (Austrapen, CSL Ltd., Australia) and incubated overnight at 37 °C. Unused cells were kept in a container of dry ice for 5 min before being returned to the −75 °C freezer (Ultralow Freezer, Nuaire, Japan).

2.2. Preparation of clarified lysate and purification of plasmid DNA

250 ml DH5α *E. coli* cells grown to produce pDNA was transferred to centrifuge bottles and centrifuged at 4600 rpm for 30 min at 4 °C. The supernatants were discarded and the bacterial pellets were resuspended in cell resuspension solution. Cell lysis solution was added with gentle mixing by inversion. Neutralization solution was then added and followed by gently inverting the centrifuge bottle several times. Centrifuged at 4600 rpm for 45 min at 4 °C to remove the precipitated floc. Finally, the clarified lysate was passed through a 0.22 μm filter to remove any particles remaining in solution after the centrifugation step. Cell lysate containing pDNA was prepared on the day it was to be used.

Pure pDNA was isolated with Promega Maxiprep DNA purification kits (Promega, USA) with purification protocols for high-copy number vectors according to the instruction of the manufacturer.

DNA concentrations were determined using optical density measurements 260 nm (OD_{260}) (Shimadzu, UV 2450, Japan). All DNA samples used had OD_{260}/OD_{280} ratios between 1.7 and 2.0.

2.3. Gel electrophoresis

The integrity of pDNA and proportion of isoforms was assessed by ethidium bromide gel electrophoresis using 0.8% agarose gels. 1 kb DNA ladder (Promega, USA) was used as a marker with fragments of 10, 8, 6, 5, 4, 3, 2, 1.5, 1 and 0.5 kbp. Ethidium bromide was used at a concentration of 0.05 μg/ml. Gels were electrophoresised at 66 V for 90 min in TAE (Tris–acetate electrophoresis buffer: 10 mM Tris, 10 mM acetate acid, 1 mM EDTA). The supercoiled DNA was analysed with densitometric scanning methods. All the gels were scanned and analysed using a Quantity One™ gel documentation system (Biorad Lab Inc., USA).

2.4. 16mer peptide

The detailed report on the structure and its binding kinetics of the selected 16mer peptide to pDNA has been explained elsewhere [29]. Briefly, 16mer peptide was synthesized by Mimotops Pty Ltd. (Melbourne, Australia). The sequence of 16mer peptide is NH_2-Cys-Met-Lys-Tyr-Val-Ser-His-Gly-Thr-Val-Ser-Arg-Val-Val-Asn-Gln-COOH, corresponding to the helixII in the structure of full DNA binding region of lac repressor protein.

2.5. Synthesis of monolith using polymerization of polyethylene glycol dimethacrylate (EDMA) and glycidyl methacrylate (GMA)

A detailed report on the chemistry involved in production of monolith and its suitablility for pDNA purification is given elsewhere [30]. In brief, we synthesized a novel poly(GMA-*co*-EDMA) monolith by first mixing EDMA as the crosslinker with GMA as the functional monomer. Then, cyclohexanol/1-dodecanol was added into EDMA/GMA mixture as an alcohol-based bi-porogen solvent in the proportion of 50/10/20/20 (cyclohexanol/1-dodecanol/GMA/EDMA/) in a total volume of 4 ml mixture. AIBN (1% weight with respect to monomer) was used to initiate the polymerisation process. The polymer mixture was sonicated for 10 min and sparged with N_2 gas to expel dissolved O_2. 4 ml of the mixture was gently transferred into a 12 cm × 1.5 cm polypropylene column (Biorad) sealed at the bottom end. The top end was sealed with a rubber bung and placed in a water bath for 18 h at 50 °C. The polymer was washed to remove all porogens and other soluble matters with methanol.

2.6. Production of lacI peptide–monolith matrix

The following procedure was used for covalent immobilisation of the lacI peptide to epoxy group of methacrylate monolith [30]. The monolith was washed with 0.5 M Na_2CO_3, 1.0 M NaCl, and pH 11.5. After removal of the washing liquid, 15 g/L of 16mer peptide ligand in binding buffer of 0.1 M Tris–HCl, pH 7.5, 0.50 M NaCl and 1 mM EDTA was added, and the column was capped and incubated for 2 h at 4 °C. Unbound material was then removed by gravity flow; the monolith was washed three times with PBS buffer and equilibrated with PBS buffer until stable baseline was reached.

2.7. Packed bed chromatographic purification

A 12 cm × 1.5 cm polypropylene column (Biorad) was packed (during the synthesis of methacrylate monolith) with 4 ml of monolith and equilibrated with 10 column volumes of PBS buffer. Feed containing the pUC19 pDNA was loaded onto the column at a flow rate of 0.5 ml/min. The column was then washed with 5 column volumes of PBS buffer and the pUC19 pDNA was eluted with PBS buffer containing NaCl. The column was operated using Duoflow biological chromatographic system (Biorad, USA). All fractions were analysed by 0.8% agarose electrophoresis and Picogreen assay.

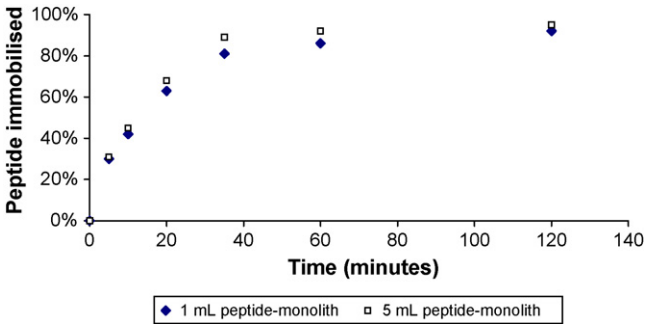

Fig. 2. 16mer peptide immobilization kinetics on monolith. The 16mer peptide immobilization kinetics is very fast and the reaction is completed within 30 min of initiation. Reaction kinetics for two different scales of operation indicates that the scale-up of the coupling process did not cause significant changes in the peptide immobilization profile.

2.8. Picogreen assay

Picogreen assay was used to determine the amount of dsDNA in a sample. Picogreen fluoresces when intercalated with dsDNA. 100 μl of sample was added to 100 μl of Picogreen reagent and 1800 μl of TE buffer. The aliquot was mixed, incubated for 30 s and then a fluorescence reading was recorded at an excitation of 480 nm and an emission of 520 nm using a spectrofluorophotometer (RF-1501, Shimadzu, Japan). The sample was diluted until fluorescence was in the linear range as determined by a calibration curve constructed using known concentrations of pDNA.

2.9. Bicinchoninic acid (BCA) protein analysis

Determination of protein levels in liquid samples was performed using BCA kit (Sigma–Aldrich, Australia) according to manufacturer's instruction, against a BSA protein calibration curve.

3. Results and discussion

Previous kinetic study has identified a 16mer peptide with suitable binding affinity for pDNA purification, which associates and dissociates with pDNA under mild conditions [29]. In this study, the feasibility of this peptide for pDNA purification is investigated using a chromatographic method.

3.1. Kinetics of peptide immobilization onto a customized monolith

Ligand density affects the binding between pDNA and 16mer peptide. At low-ligand densities, the monolith fails to bind pDNA; at intermediate ligand densities, pDNA binds but is subsequently lost during column wash operations; at high-ligand densities, pDNA is retained completely through the loading and washing but has to be eluted with higher salt concentrations. In order to optimize the ligand density on the monolith matrix, the reaction rate during the coupling process was studied using 16mer peptide immobilization on the monolith. The reaction is nearly complete in 40 min as shown in Fig. 2, so the kinetics of the immobilization is slow and need to be considered during immobilization.

3.2. Packed bed adsorption of pure plasmid pUC19 to 16mer peptide

0.06 mg 16mer peptide was immobilised on a 4 ml stationary monolith in a binding buffer. After washing the monolith with 0.50 M $NaHCO_3$ and DI water, 200 μl pDNA (501 μg/ml) was applied to the column equilibrated with 0.01 M PBS buffer, pH 7.4. Then, the column was washed with 0.01 M PBS buffer, pH 7.4 and followed by the subsequent elution of bound pDNA with 0.01 M PBS buffer containing 1 M NaCl. A flow rate of 0.5 ml/min was applied for loading and washing steps and 1 ml/min for elution. The $OD_{260\,nm}$ and buffer B signal from Duoflow biological chromatography were plotted against elution time in Fig. 3. pDNA concentration and purity were determined by absorbance at 260 and 280 nm using a UV-2450 spectrophotometer and Picogreen assay. DNA concentration was determined using the following formula: $A_{260} \times$ dilution factor $\times$ 50 μg/ml (extinction coefficient), purity was determined by A_{260}/A_{280} ratio. Agarose electrophoresis gel was also used to check whether there were residual RNA and bacterial chromosomal DNA in the pDNA product.

It is evident in Fig. 3 that there is only a single peak during the prolonged elution phase. This indicates that there exists only one specific binding in this chromatographic system, which peptide ligand has sufficient strength to capture and elute pDNA.

The ethidium bromide agarose electrophoresis gel (the inset image in Fig. 3) shows that pUC19 plasmid was absorbed and eluted without any degradation. Densitometry analysis of the gel bands showed that the elution fraction was 92% supercoiled pDNA. This indicates that 16mer peptide has demonstrated its preference for supercoiled pDNA over linear and open circular form of pDNA.

Picogreen assays showed some pDNA (65.1 ± 2.5 μg) in the flow through and washing as presented in Fig. 3. This indicates that peptide–monolith support was saturated with pUC19 plasmid. Subsequent elution using 0.01 M PBS buffer containing 1.0 M NaCl resulted in a peak containing 28.0 ± 2.1 μg pDNA. According to mass balance, the amount bound was 34.9 ± 2.1 μg. The elution yield of the pDNA bound was 80.1 ± 5.5%. This has led to a dynamic capacity of 21.6 ± 4.5 μg pUC19/ml monolith. pDNA was eluted in almost physiological pH (below pH 8.0), which is good to be able to directly elute pDNA into a formulation buffer that requires no further processing.

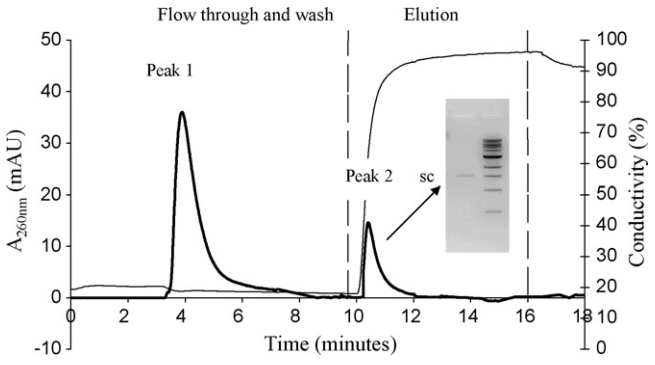

Fig. 3. Chromatogram of plasmid pUC19 loaded on 16mer peptide immobilised on a 4-ml customized monolith. Bound plasmid DNA was eluted using PBS buffer (0.01 M PBS, KCl 0.0027 M; pH 7.4) with additional NaCl (final concentration: 1 M). The $OD_{260\,nm}$ results from Duoflow biological chromatographic unit (flow rate 0.5 ml/min) are plotted against the left *y*-axis and the conductivity of the liquid in the system is plotted against the right *y*-axis. Absorbance (260 nm) is shown by the solid line and conductivity by the fine line. The fraction was collected and run on an ethidium bromide electrophoresis gel against 1 kbp DNA ladder as shown in the inset picture.

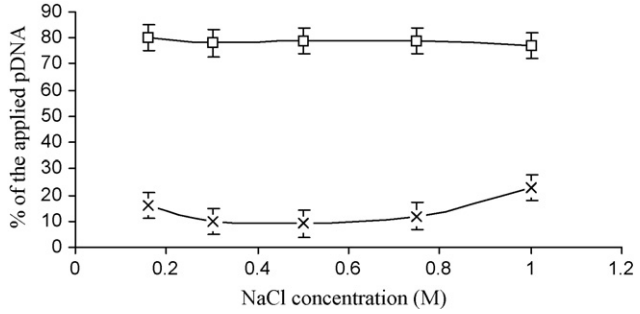

Fig. 4. Affinity chromatography at different NaCl concentrations. The column was equilibrated with 0.01 M PBS buffer, pH 7.4 at a flow rate 0.5 ml/min. After 200 μl of pDNA was applied onto the column, the column was flowed at a flow rate of 1.0 ml/min, first with the equilibrium buffer to wash out non-adsorbed pDNA (□, flow through of pDNA unbound), then with 0.01 M PBS buffer containing different NaCl concentrations (0.158, 0.3, 0.5, 0.75 and 1.0 M) to elute bound pDNA (×, dissociation of pDNA bound). All data are expressed as percentages of the amounts of the applied pDNA, calculated from Picogreen assay.

3.2.1. Effect of sodium chloride

Experiments were performed at different concentrations of sodium chloride, the results of which are presented in Fig. 4. The column was equilibrated with 0.010 M PBS buffer, pH 7.4 at a flow rate 0.5 ml/min. 200 μl of pDNA was then loaded onto the column. At a flow rate of 1.0 ml/min, the column was first washed with the equilibrium buffer, then with 0.01 M PBS buffer containing different NaCl concentrations (0.158, 0.3, 0.5, 0.75 and 1.0 M) to elute bound pDNA. All data are expressed as a percentage of the initial amount of the applied pDNA, calculated via the Picogreen assay. The results (Fig. 4) demonstrate that the highest recovery was obtained for a NaCl concentration of 1.0 M.

Table 1
Effect of NaCl concentrations on electrostatic and hydrophobic interaction of plasmid pUC19 for 16mer peptide

	Concentration of NaCl (M)		
	0	0.5	1
Electrostatic interaction	Strongest[a]	Medium[a]	Weakest[a]
Hydrophobic interaction	Weakest[a]	Medium[a]	Strongest[a]
% of the applied pDNA (eluted)	16	9	23

Monolith was overloaded with 200 μg of pDNA ($n = 2$) with the average of the runs displayed.

[a] Only compare them among NaCl concentrations tested.

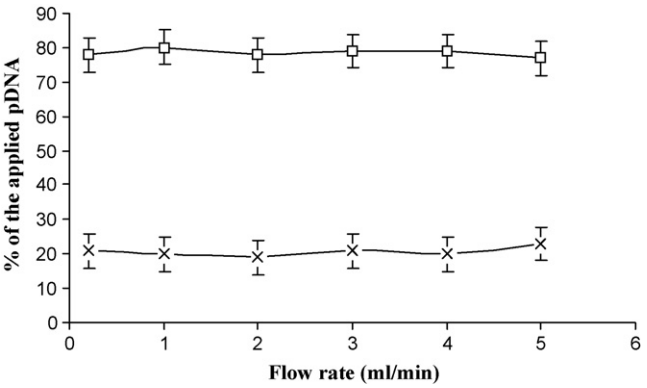

Fig. 5. Effect of flow rate on the binding of the pDNA to 16mer peptide immobilised on the monolith. For basic experimental conditions same as in Fig. 3. Elution buffer: 0.01 M PBS buffer containing 1.0 M NaCl. Flow rates for washing out of unbound (□, flow through of pDNA unbound) and elution of bound pDNA (×, dissociation of pDNA bound): 0.2, 1.0, 2.0, 3.0, 4.0 and 5.0 ml/min accordingly.

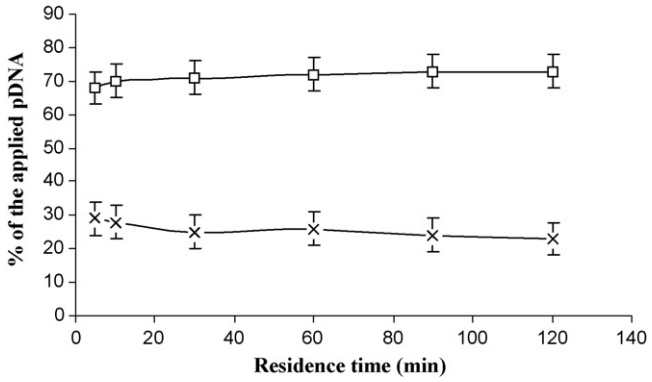

Fig. 6. Effect of pDNA residence time in the column on the binding of the pDNA to 16mer peptide. 5 min after application of 200 μl of sample, the column was eluted with 0.01 M PBS buffer containing 1.0 M NaCl (residence time = 5 min). The experiment was repeated with the exception that the flow was stopped for 10, 30, 60, 90 and 120 min before elution started (□, flow through of pDNA unbound; ×, dissociation of pDNA bound).

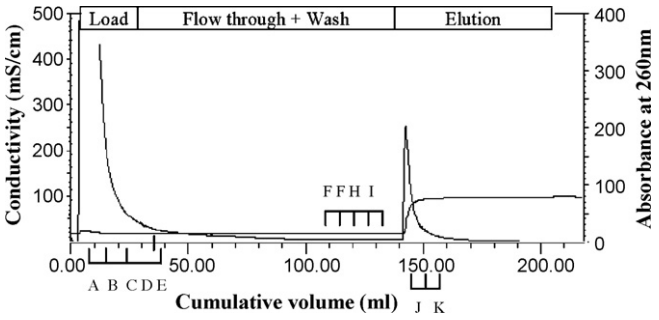

Fig. 7. Chromatogram showing purification of pDNA from *Escherichia coli* DH5α–pUC19 clarified lysate using 16mer peptide as an affinity ligand on 4 ml monolith. Polypropylene column 12 cm × 1.5 cm packed with 4 ml fictionalized monolith was connected with a movable adaptor and configured to Biorad Duoflow biological chromatographic system. Chromatographic purification of pDNA was performed using 0.01 M PBS buffer, pH 7.4 as buffer A and 0.01 M PBS buffer containing 1 M NaCl as buffer B. 1 ml of clarified lysate was diluted (0.5×) with buffer A and applied at 0.5 ml/min. Only a single peak was observed during the whole elution phase.

Affinity is often a combination of electrostatic, hydrophobic and hydrogen bond interaction. Electrostatic interaction decreases with an increase in ionic strength, but hydrophobic interaction increases. As shown in Table 1, the affinity of pDNA to the 16mer peptide may be determined by the weaker interaction of either electrostatic or hydrophobic at different NaCl concentrations. NaCl concentration has a stronger effect on electrostatic interaction than hydrophobic interaction.

3.2.2. Effect of flow rate

The experiments were performed in a 0.01-M PBS buffer containing 1.0 M sodium chloride. The contributions from hydrophobic interaction to the binding between peptide and pDNA are much stronger than those from electrostatic interaction. As shown in Fig. 5, no significant change was observed on the percentage of washing out or eluted pDNA of higher flow rates. This indicates that the binding between peptide and pDNA displays adsorption–desorption fast kinetics: there is more time for pDNA to associate and dissociate at low-flow rates than at high-flow rates, but this does not significantly affect the binding between pDNA and peptide as the binding is not diffusion limited. The combination of a convection controlled fluidic system, such as a monolith, with the fast kinetics of this affinity mechanism, shows great promise as a viable method for pDNA purification.

3.2.3. Effect of sample residence time in the column

The results are presented diagrammatically in Fig. 6 (see the legend for details in the experiment). The percentages of eluted and washed out pDNA exhibited no significant change during any pDNA residence time in the column. This indicates that the binding between pDNA and 16mer peptide is fast kinetics.

3.3. Packed bed adsorption of pUC19 from a clarified lysate

The feasibility of 16mer peptide for pDNA purification was further validated using a clarified lysate. The clarified lysate was loaded onto the column to determine whether pUC19 could be purified from a feed containing contaminants gDNA, RNA and protein. The elution buffer was PBS buffer with additional NaCl. Ten runs were performed using the same monolith to determine whether the lacI peptide–monolith could be regenerated for use. The following elution strategies were taken to eliminate the RNA contamination: (1) extend washing time, (2) increase flow rate, (3) linear elution strategy, (4) increase salt concentration in the elution buffer, (5) reduce sample loading to avoid the clogging of the monolith. Chromatogram of the clarified lysate loaded on 4 ml 16mer immobilised monolith was presented in Fig. 7. The fractions are collected and examined by electrophoresis gel as shown in Fig. 8.

An ethidium bromide agarose electrophoresis gel shows that purified pUC19 product appears predominantly as supercoiled pDNA with below detectable concentrations of RNA (lane 1 in Fig. 8). The fraction of supercoiled pDNA increased from 60% to 96% as measured via densitometric analysis. This substantiates that pUC19 plasmid was captured and eluted from the clarified lysate. Gel analysis also shows that some pUC19 was lost in the flow through. Total protein concentration was 195 μg/ml in the

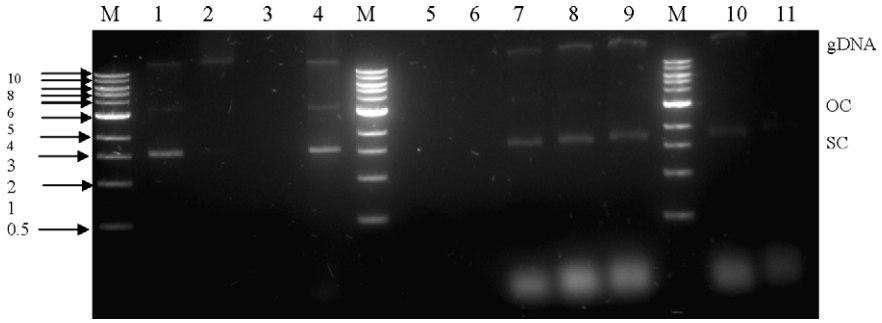

Fig. 8. Agarose electrophoresis gel of a clarified lysate containing pUC19 plasmid using 16mer peptide immobilised on a 4-ml monolith. M: 1 kb DNA marker; lanes 1 and 4: elution fraction J and K; lanes 2, 3, 5 and 6: end of the washing phase F, G, H and I; lanes 7–11: flow through A, B, C, D and E as marked in Fig. 7.

clarified lysate measured using BCA protein assay, but was below detectable levels in the purified plasmid fraction.

3.4. Cleaning and reuse of peptide–monolith support

The stability of the 16mer peptide monolith support was evaluated by using the same column for 16 consecutive runs. The repeated runs were maintained within small ranges of load mass and composition (±9.5% change). There was no decrease in performance over its lifetime, as determined by pDNA yield. Periodic blank elutions were performed during the study and analysed for pDNA carryover. A very low level of pDNA (<0.01%) could be detected by agarose electrophoresis gel in the blank elution pool and was consistent from run to run.

4. Conclusions

A single step purification of pDNA has been developed and validated with both pure pUC19 plasmid and clarified lysate from *E. coli* DH5α–pUC19. A customized monolith was first synthesized via free radical liquid porogenic polymerisation of EDMA and GMA. The selected 16mer peptide was chemically synthesized, and the affinity matrix was then produced by coupling 16mer peptide to poly(GMA-*co*-EDMA) monolith. The column packed with peptide–monolith was connected to a biological chromatographic unit. After validation with pure pDNA, a clarified lysate containing RNA, gDNA and protein was applied in the chromatography. There was only a single peak during the whole elution phase, which indicates that pDNA was successfully captured and eluted from the clarified lysate in a single step. The purified pDNA was examined by DNA electrophoresis and restriction analyses. The results demonstrate that 81% of pDNA was recovered with a purity of 92% and contaminants gDNA, RNA and protein were removed to the limit of the detection. As it can eliminate some steps in the chromatographic purification process, the single stage purification method developed has the potential to improve overall plasmid recovery, processing time and cost.

For the purpose of process optimization, the effects of NaCl concentration, flow rates and residence time in the column were also studied. It was found that the affinity of pDNA for 16mer peptide may be controlled by the weaker one of electrostatic and hydrophobic interaction, and NaCl concentrations may have a stronger effect on electrostatic interaction than on hydrophobic interaction. Flow rates and pDNA residence time in the column have little effect on the binding between pDNA and 16mer peptide.

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