Application of a new hybrid organic–inorganic monolithic column for efficient deoxyribonucleic acid purification

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ABSTRACT

A new hybrid organic–inorganic monolithic column for efficient deoxyribonucleic acid (DNA) extraction was prepared in situ by polymerization of N-(β-aminoethyl)-γ-aminopropyltriethoxysilane (AEAPTES) and tetraethoxysilane (TEOS). The main extraction mechanism was based on the Coulombic force between DNA and the amino silica hybrid monolithic column. DNA extraction conditions, such as pH, ion concentration and type, and loading capacity, were optimized online by capillary electrophoresis with laser-induced fluorescence detection. Under optimal condition, a 6.0-cm monolithic column provided a capacity of 48 ng DNA with an extraction efficiency of $74 \pm 6.3\%$ ($X \pm$ RSD). The DNA extraction process on this monolithic column was carried out in a totally aqueous system for the successful purification of DNA and removal of proteins. The PBE2 plasmid could be extracted from Bacillus subtilis (B. subtilis) crude lysate within 25 min, and the purified DNA was suitable for the amplification of a target fragment by polymerase chain reaction. This study demonstrates a new attractive solid-phase support for DNA extraction to meet the increasingly miniaturized and automated trends of genetic analyses.

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1. Introduction

The purification of deoxyribonucleic acid (DNA) from biological samples is the most important step in many genetic profiles and clinical applications. It is widely acknowledged that a robust DNA preparation method is a key to ensuring accurate amplification of a target DNA, and the purity of the DNA samples is critical to obtain high resolution in different genotyping strategies [1]. For this reason, great attention has focused on the development of fast and robust DNA isolation methods, including organic solvent extraction, ion exchange chromatographic and solid-phase adsorption-based purification systems, to enhance sample quality and facilitate automation [2]. Among the various DNA purification protocols, solid-phase extraction (SPE) is the most promising technique to handle forensic and clinical samples in terms of low reagent consumption, small volume requirements and short analysis time. In addition, this technique is also amenable to integrating into the micro-total analysis system (μ-TAS) [3].

While the selection of solid-phase support is one of the most important factors for the SPE of DNA, supports that can be applied in a microscale format for DNA extraction are relatively few, and this lack of choices has hampered the development of genetic analyses. To prepare high-quality DNA samples for subsequent genetic processes, such as polymerase chain reaction (PCR), genetic recombination and

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clinical hybridization, new types of DNA extraction supports with high capacity and productivity for DNA purification in a microscale format are required.

Silica-based particles have been the most widely used solid-phase support for DNA extraction. These supports have been used in a variety of forms, including silica beads [4], sol–gel hybrid silica beads [5], and sol–gel silica monolithic columns [6,7]. During the purification procedure, DNA molecules are selectively adsorbed on the silica-based support in high concentrations of chaotropic salt (e.g., guanidine hydrochloride, guanidine thiocyanate) solutions. Proteins and other contaminants in biological samples are washed away by organic solvents (e.g., ethanol, propanol). The adsorbed DNA is then eluted with the solutions of low salt concentrations [8]. Silica-based extraction protocols represent the most widely used and accepted methods for conventional DNA purification from biological sources, with the respect to extraction speed and efficiency. However, because high concentrations of chaotropic salt solutions and organic solvents are used in the extraction procedure, the existence of these reagents in the eluted fraction may have negative effects on the subsequent genetic process (e.g., PCR analysis). Witek et al. reported that high concentrations of sodium chloride (>50 mM) or trace amounts of ethanol (1.7%, $v v^{-1}$) in the eluted fraction could dramatically decrease the efficiency of PCR amplification [9]. As a result, the use of these reagents in the extraction procedure may create many problems in the subsequent DNA analysis.

Ideal solid-phase supports amenable to DNA SPE in a miniaturized format would be those that not only provided high yields of DNA, but also low concentrations of salts and other inhibitors in the eluted fraction. Several solid-phase supports have been tested to address these problems in DNA purification. Nakagawa et al. used amino silane to coat the open channel in a chip to create the solid-phase support for DNA extraction [10]. Because the adsorption of DNA on this support was primarily based on Coulombic forces between DNA and the amino groups, DNA purification could be readily achieved by changing the solutions from acidic to basic. In a similar approach, Cao et al. coated chitosan on 64 parallel channels in a microchip for effective DNA capture and release [11]. Since only aqueous solutions were used in the extraction procedure, inhibitors, such as high concentrations of chaotropic salts and organic solvents, that might affect the subsequent DNA analysis were avoided. Thus, these solid-phase supports are particularly compatible with the subsequent DNA analysis. While these supports show some potential, special microchip designs are required to enhance the effective surface area to increase the capacity for DNA, because of the open tubular channel form used. Consequently, the fabrication cost of the microchip increased, which limits their applications in many laboratories.

Several approaches have been suggested to increase the surface area of the solid-phase support to enhance the capacity for DNA. Examples include the use of smaller size particles or monoliths. Decreasing the particle size has proved to be an effective method to increase the surface area for DNA adsorption [12]. However, a too small size of the particle can cause extremely high back pressure during the extraction procedure. The monotonic decrease of the size of particles to increase the surface area of the solid-phase support has consequently been limited in practical application. At the same time, it is also inconvenient to pack the bed or frits in a microchip channel. Monolithic beds, on the other hand, offer an effective solution to increase the surface area with low back pressure for DNA extraction. A monolith is a continuous piece of highly porous material, typically created by *in situ* polymerization of monomers and characterized by a bimodal pore structure, consisting of large flow-through pores for high permeability and small diffusion pores for the desired high surface area to provide a high capacity [13]. Owing to this unique structure, solute transport in a monolith is dominated by convection, instead of diffusion as observed in conventional particulate chromatographic media. Because the skeleton is covalently bonded to the inner walls of the fused-silica channels, monolithic columns do not require retaining frits that are difficult to reproducibly fabricate and often result in bubble formation in packed columns [14]. The special structure of the monolithic column is particularly favorable for DNA purification. Although various types of monolithic columns have been applied for macro-scale DNA purification [15–18], their application for micro-scale DNA purification is much less common [6,7]. To date, the potential of the monolithic column for DNA purification in a miniaturized format has not been fully demonstrated.

In the present work, a new hybrid organic–inorganic monolithic column was first prepared *in situ* by polymerization of tetraethoxysilane (TEOS) and N-(β-aminoethyl)-γ-aminopropyltriethoxysilane (AEAPTES) for efficient DNA purification. The extraction procedure was carried out in a totally aqueous system. Organic solvents and high concentrations of salts were avoided. As a result, the DNA purification method would fulfill the requirements of μ-TAS to provide high-quality DNA for genetic analysis. Extraction conditions, such as pH, ion concentration and type, and loading capacity, were optimized online for effective DNA purification. DNA extraction from *Bacillus subtilis* (*B. subtilis*) crude lysate using the hybrid monolithic column and subsequent PCR using the recovered DNA was examined to assess the potential application of the column.

2. Experimental

2.1. Chemicals and materials

TEOS and AEAPTES were purchased from Wuhan University Silicone New Material (Wuhan, China). Cetyltrimethylammonium bromide (CTMAB), ethanol, sodium dihydrogenphosphate (NaH$_2$PO$_4$), disodium hydrogenphosphate (Na$_2$HPO$_4$), tris(hydroxylmethyl)aminomethane (tris) and ethylenediaminetetraacetic acid (EDTA) were analytical grade from Shanghai Reagent Factory (Shanghai, China). 2-(N-Morpholino)-ethanesulfonic acid (Mes) was bought from Amresco (Solon, OH, USA). Hydroxyethylcellulose (HEC) was from Serva (Heidelberg, Germany). Herring sperm DNA was purchased from Sigma (St Louis, MO, USA). Sybr green I was obtained from Molecular Probes (Eugene, OR, USA). Proteinase K was purchased from Merck (Röhm, Darmstadt, Germany). DL 2000 DNA marker, PCR buffer, deoxynucleotide triphos-

phates (dNTPs), and Taq polymerase were from TaKaRa Biotech. (Dalian, China). PCR primers were from Shanghai Boya Biotech. (Shanghai, China). The BCA protein assay kit was from Beyotime Inst. Biotech. (Haimen, China). TE buffer (10–500 mM Tris, 2 mM EDTA, titrated to pH 7.0 with 1.0 M HCl), NaCl solution (10–500 mM NaCl, dissolved in 2 mM TE buffer at pH 7.0) and sodium phosphate solution (10–500 mM Na_2HPO_4, titrated to pH 9.0 or 10.0 with 1.0 M NaOH) were used for the SPE procedures. For the optimization of loading conditions, the pH values (6.0–11.0) of TE solutions (10 mM Tris, 2 mM EDTA) were adjusted with 1.0 M HCl or 1.0 M NaOH, and NaH_2PO_4–Na_2HPO_4 solutions at pH 7.0 were prepared by mixing equivalent concentrations (500 mM) of NaH_2PO_4 and Na_2HPO_4 solutions. HEC (0.20%, w v^{-1}) in TME buffer (40 mM Tris, 40 mM Mes, 2 mM EDTA, pH 6.55) was used for the separation of DNA by capillary electrophoresis (CE). Sybr green I with diluted concentration of 1:10,000 was added to the TME buffer for the intercalation of DNA prior to separation.

2.2. Equipment

Morphology of the monolithic column was examined by Quanta 200 scanning electron microscopy (SEM) (Philips-FEI, Eindhoven, the Netherlands) system. PCR was performed in a PTC-100 Peltier thermocycler (Bio-Rad, Hercules, CA, USA). DNA and protein quantifications were carried out using a Spectra max M2 multifunctional microplate reader (MDC, Sunnyvale, CA, USA). DNA extraction performance and DNA separation were carried out in the laboratory-built CE system with laser-induced fluorescence (LIF) detection. A Cr-YAG laser with 473 nm output (Linyun Photoelectric System, Wuhan, China) was used for excitation. A narrow-band interference filter at 520 nm was used to block stray light. The signal from the photomultiplier tube was fed into an EC 2000 chromatography workstation (Dalian Elite Analytical Instruments, Dalian, China) and then stored on personal computer. A 42-cm (35 cm to the detector) uncoated fused-silica capillary (Yongnian Optical Fiber Factory, Hebei, China) with 75 μm i.d. and 375 μm o.d. was used for DNA separation.

2.3. Preparation of B. subtilis plasmid DNA

B. *subtilis* harboring the PBE2 plasmid (7.8 kbp) that contained a cloned 1.6-kbp fragment was grown overnight in LB medium (L^{-1}: 10 g tryptone, 5 g yeast extract, 5 g NaCl) in the presence of kanamycin (300 μg mL^{-1}) and was harvested by centrifugation (12 000 rpm, 1 min). Cells were resuspended in water to an approximate density of 10^9 cells mL^{-1}, and lysed with lysozyme (100 mg mL^{-1}). A volume of 100 μL B. *subtilis* cells were pipetted into a vial from the cultured medium. The cells were lysed with 25 μL Triton-X 100 (10%, w v^{-1}) and 15 μL proteinase K (10 mg mL^{-1}). After incubation at room temperature for 10 min, the mixture was heated to 37 °C for 30 min to ensure complete lysis. The lysate was diluted 20 times in 50 mM TE buffer (pH 7.0) prior to extraction.

2.4. Preparation of the hybrid monolithic column

Fused-silica capillary with 250 μm i.d. and 370 μm o.d. was used to prepare the monolithic column. The capillary was
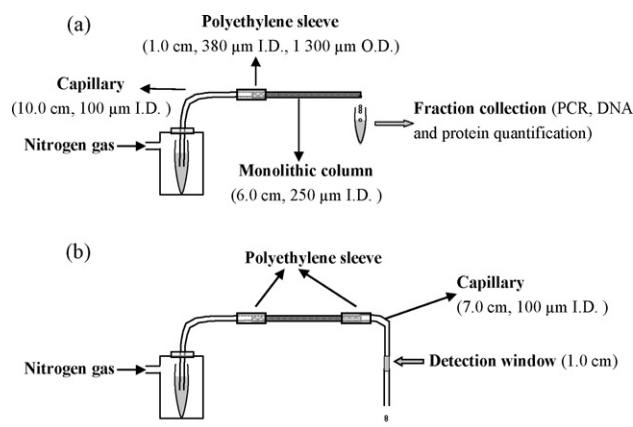
Fig. 1 – Schematic diagram of the prepared SPE device: (a) off-line SPE device and (b) online SPE device coupled to the CE instrument.

rinsed with 1.0 M NaOH for 12 h, 1.0 M HCl for 12 h, water for 1 h, and ethanol for 1 h. The flow rate of these wash solutions was controlled to 600 μL h^{-1} by pumping the solutions via positive pressure supplied by the nitrogen cylinders with a gas valve (Fig. 1a). After drying under a stream of nitrogen for 3 h, the pretreated capillary was stored at 40 °C.

The monolithic column was prepared as follows: 225 μL ethanol, 75 μL water and 0.0222 g CTMAB were mixed in 1.5 mL eppendorf tube and ultrasonicated for 5 min at 0 °C. A volume of 160 μL TEOS and 40 μL AEAPTES were then added to the above mixture and ultrasonicated for 30 s at 0 °C. Finally, the homogenous solution was filled into the pretreated capillary via positive pressure supplied by the nitrogen cylinders with a gas valve and stored at 40 °C to form the monolithic column. Gelation occurred within 10 min and the gel was subsequently aged in the capillary overnight at the same temperature. The monolithic column was then transferred to 70 °C for another 24 h to accelerate the evaporation of ethanol and the unreacted silica monomer. Before use, the monolithic column was rinsed with ethanol and water for 30 min, respectively.

2.5. Preparation of the SPE device

Fig. 1 is the schematic diagram of the prepared SPE device. A 6.0-cm monolithic column was used for DNA extraction in all extraction procedures. A 1.0-cm polyethylene sleeve (380 μm i.d., 1300 μm o.d.) was used to connect the monolithic column and a 10-cm unreacted fused-silica capillary (100 μm i.d. and 375 μm o.d.). Polyimide glue was applied to hold the polyethylene sleeve and the capillaries in place. The samples and wash solvents were introduced by pumping the solutions via positive pressure supplied by the nitrogen cylinders with a gas valve (Fig. 1a). The flow rate of the solutions was evaluated from the time needed to push a constant volume through the device when a constant pressure was applied. Based on the measured flow rate of each solution, pressure was changed to ensure the constant flow rate in the extraction procedure. The fractions from the SPE device were collected into microcentrifuge tubes for the following analysis (such as PCR, DNA and protein quantifications). As shown in Fig. 1b, the online

SPE device was prepared by connecting the other end of the monolithic column to a 7.0-cm unreacted fused-silica capillary (100 μm i.d. and 375 μm o.d.) using another polyethylene sleeve. A 1.0-cm detection window was opened in the middle of the capillary by removal of the polyimide coating for online monitoring the change of fluorescence. The prepared extraction device was then assembled on the laboratory-built CE instrument to assess the DNA extraction performance.

2.6. DNA extraction procedure

DNA extraction generally involved three steps: load, wash and elution. During the extraction procedure, the flow rate of the solutions was controlled to 240 μL h^{-1}. Before extraction, the SPE device was equilibrated with 50 mM TE buffer (pH 7.0) for 5 min. DNA samples diluted in TE buffer were loaded on the column by pressure injections for various injection times. A wash step was subsequently performed by the same TE buffer to remove unbond DNA and contaminants. DNA was then eluted with 200 mM sodium phosphate solution (pH 10.0). During the consecutive extractions, the monolithic column was washed with 500 mM sodium phosphate solution (pH 10.0) and water for 5 min each to regenerate the extraction bed. For the online detection of the DNA extraction procedure in CE instrument, Sybr green I with diluted concentration of 1:10 000 was added to all solutions to indicate the change of fluorescence.

2.7. DNA and protein quantifications

The load, wash, and elution solutions were collected for DNA and protein quantifications. The amount of DNA was detected using Sybr green I ($\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm) in multifunctional microplate reader. The amount of protein was detected using the BCA protein assay kit at 562 nm in the same microplate reader.

2.8. PCR procedure

PCR was performed in a total volume of 25 μL containing 10× PCR buffer, 0.5 μL dNTPs (each 10 mM in 10 mM Tris–HCl buffer, pH 7.5), 0.2 μL Taq polymerase (5 U μL^{-1}), and 1 μL primers (10 μM). Thirty rounds of temperature cycling were performed in the thermocycler with denaturation at 94 °C, primer annealing at 56 °C, and elongation at 72 °C (each for 30 s). This was followed by 10 min incubation at 72 °C. A volume of 1 μL crude lysate and an equivalent amount of purified PBE2 plasmid DNA extracted by the monolithic column were used directly for PCR amplification.

3. Results and discussion

3.1. Preparation of the hybrid monolithic column

The development of an effective solid-phase support for DNA extraction played an importance role in coupling with the μ-TAS for genetic analysis. Because monolithic column could provide high surface area for DNA adsorption and low back pressure for extraction solutions to pass through, it seemed to be an attractive candidate for DNA purification. However,
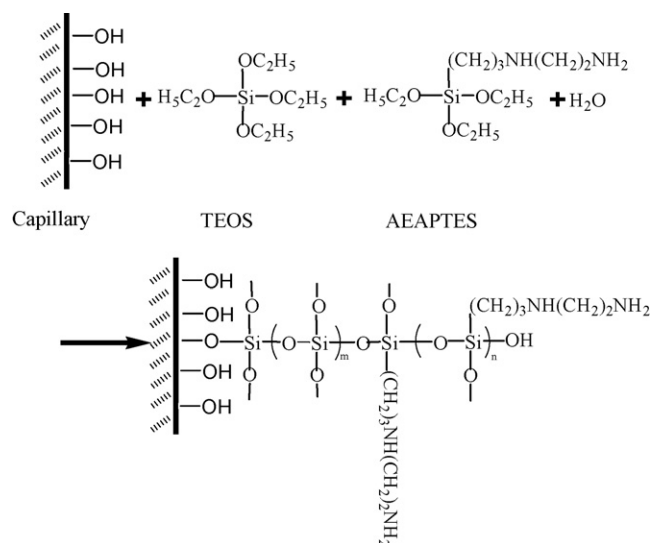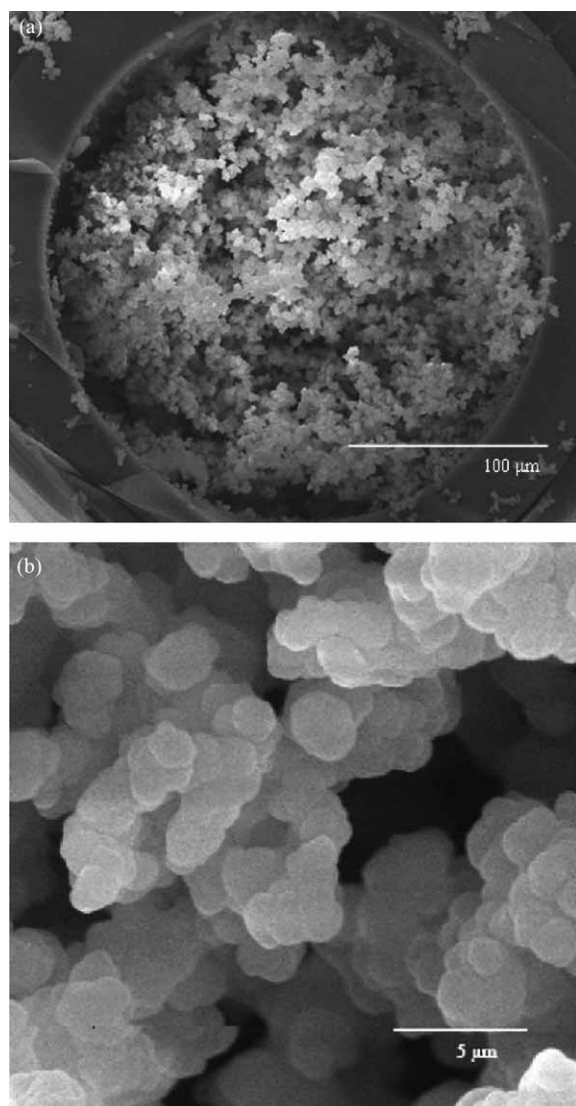

Fig. 2 – Schematic diagram of the sol–gel chemical reaction for the preparation of the monolithic column.

column preparation using large diameter capillaries was difficult because of the shrinkage of the silica skeletons. Since the combination of the different types of silica monomers to form the monolithic column could not only decrease the shrinkage of the skeletons [19,20], but also avoid additional steps of introducing the functional groups for DNA adsorption, TEOS and AEAPTES were selected to form the adsorption phase. A schematic diagram of the complete sol–gel reaction in the capillary is shown in Fig. 2. Under optimal condition, TEOS and AEAPTES underwent hydrolysis in the presence of water, where the alkoxy groups were converted into hydroxyl groups. As the reactions proceeded, gelation started with aggregation of the polymers into fractal clusters. Then, the clusters interpenetrated, to some extent, and finally linked together to form the network. Because the silanol groups on the inner wall of the capillary can also chemically bond with the polymeric sol–gel network, a continuous bed was formed within the capillary. The SEM photograph in Fig. 3a indicates that the silica skeletons formed could attach to the inner wall of the capillary. No large void was observed along the wall. The clusters of uniform-sized silica skeletons of about 2.0 μm and through-pores sizes up to 5.0 μm were uniformly distributed on the monolith (Fig. 3b). The small-sized silica skeletons and relatively large through-pores could provide high extraction capacity and good permeability. The long-term stability of the prepared column was confirmed by 10 consecutive DNA extractions, and no shatter of the extraction bed was observed during the extraction procedures with different extraction solutions.

3.2. Optimization of the loading conditions

Because the initial adsorption behavior of DNA on the hybrid monolithic column determined both the extraction efficiency and extraction speed, much attention was focused on the loading conditions for DNA extraction. Since the CE instrument with LIF detection can provide an excellent platform to
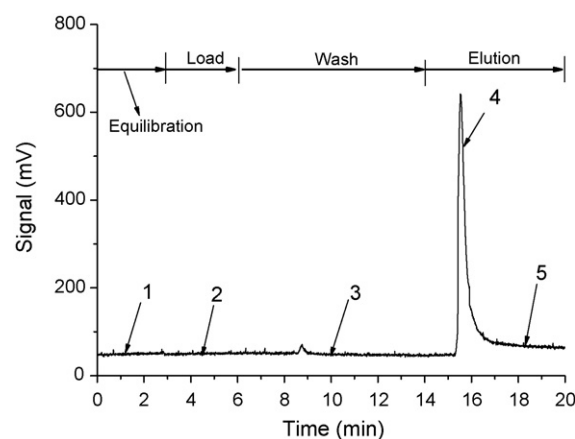
Fig. 4 – Typical extraction profile of herring sperm DNA stained with Sybr green I on the monolithic column: (1) equilibrium baseline (10 mM TE buffer, pH 6.0), (2) load step baseline (2 ng μL^{-1} herring sperm DNA in 10 mM TE buffer, pH 6.0), (3) wash step baseline (10 mM TE buffer, pH 6.0), (4) elution peak (herring sperm DNA) and (5) elution step baseline (200 mM sodium phosphate solution, pH 9.0). Flow rate: 240 $\mu L\,h^{-1}$.

Fig. 3 – SEM images of a cross section of the monolithic column: (a) 450× magnification and (b) 6000× magnification.

evaluate the DNA extraction profiles online, in terms of its high sensitivity and simplicity, loading conditions, such as pH, ion concentration and type, were optimized using this device. Fig. 4 is a typical extraction profile of herring sperm DNA stained with Sybr green I on the hybrid monolithic column. Parts 1, 2, 3, 5 of the profile in Fig. 4 are the baselines of different solutions with different optical properties used in equilibrium, load, wash and elution steps, respectively. Part 4 of the profile in Fig. 4 is the elution peak of herring sperm DNA. The adsorption performances of DNA under different loading conditions were evaluated by comparing the elution peak areas at a constant flow rate.

The effect of pH on the DNA retention behavior was investigated by changing the pH of the loading solutions from 6.0 to 11.0. As shown in Fig. 5, the elution peak areas of DNA in the loading solutions with pH ranging from 6.0 to 9.0 showed little change, which corresponded well with the baseline changes observed in the load and wash steps under these pH condi-

tions. The elution peak areas of DNA in the loading solutions with pH values above 10.0 decreased dramatically, indicating that a large percentage of the loaded DNA was lost in this pH range. The DNA extraction profiles presented in Fig. 5a also confirmed these changes. The baselines in the wash steps increased when the pH value was above 10.0. These results revealed that the pH of loading solutions greatly affected the adsorption behavior of DNA on the hybrid monolithic column. Balladur et al. reported that the amount of adsorbed DNA oligonucleotide on an aminopropylsilane-modified wafer decreased with the increased pH of the adsorption solution [21]. Our results were consistent with this report. The zeta potential of the silica nanoparticles modified by AEAPTES also suggested that the particles were positively charged in the pH range from 4.0 to 8.6 and became negatively charged under more alkaline condition [22]. The free silanol groups of the monolith were in the SiOH form under acidic conditions, and the amino groups on the monolithic column could carry cationic charges. Thus, negatively charged DNA interacted well with the positively charged monolithic column. Then, under alkaline conditions, the Coulombic force between DNA and the column became weaker because of the decrease of positively charged amino groups and the increase of negatively charged silanols (SiO^-). As a result, the adsorbed amount of DNA on the amino silica hybrid monolithic column decreased with the increase of pH. This understanding of the pH dependence of DNA adsorption on the monolithic column was important for DNA extraction. The extraction procedure thus could be realized by changing the pH of the solutions for effective DNA adsorption and desorption. Because neutral condition may prevent the possible precipitation of proteins and lipids in biological samples from clogging the monolithic column, pH 7.0 was selected as the optimal condition for effective DNA loading. From the results, it could also be concluded that DNA was more easily desorbed at higher pH. Neverthe-
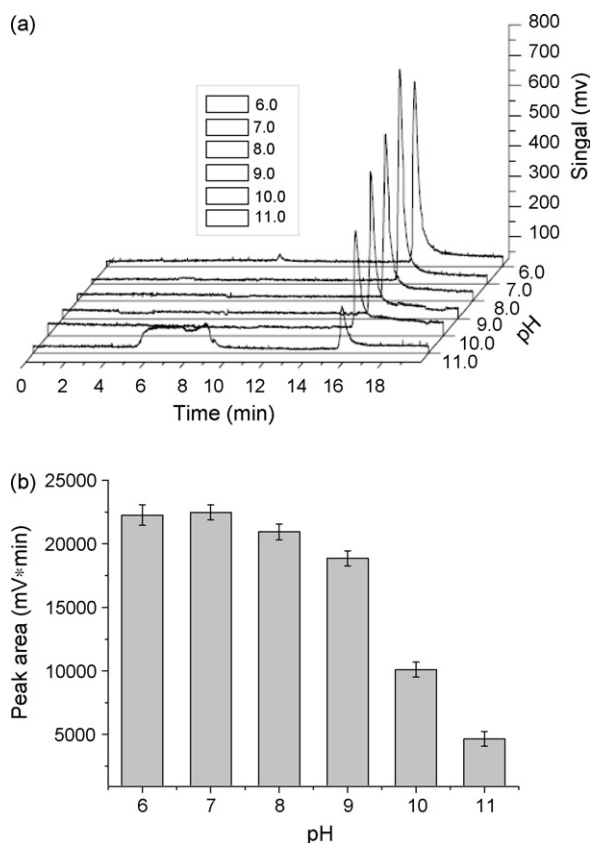
Fig. 5 – Effect of pH on the adsorption behavior of herring sperm DNA on the monolithic column: (a) extraction profile of herring sperm DNA in the loading solutions at different pH and (b) effect of pH on the elution peak area of herring sperm DNA. Load condition: 2 ng μL⁻¹ herring sperm DNA in 10 mM TE solution at different pH. Other conditions are as the same as in Fig. 4.

less, extremely high pH conditions may decrease the stability of DNA [23]. Thus, pH 10.0 was chosen as the elution condition without further optimization.

In addition to the effect of pH on the DNA adsorption, the retention behavior of DNA in different solutions at pH 7.0 was also investigated. As illustrated in Fig. 6, the DNA elution peak areas in TE, NaCl and Na_2HPO_4–NaH_2PO_4 loading solutions decreased with increased salt concentrations. The phosphate anion had the most significant effect on the adsorption of DNA. Only 2.42% of the elution peak area was observed in 500 mM Na_2HPO_4–NaH_2PO_4 solution compared with that in 10 mM solution. The TE and NaCl solutions had similar influence on the retention behaviors of DNA, except that the elution peak area in the former was somewhat larger than that in the latter. Regarding different salts at the same concentration, the competitive ability of Na_2HPO_4–NaH_2PO_4 solution was the most powerful while TE buffer was the weakest. These results could be explained by the Coulombic adsorption mechanism of DNA on the amino silica hybrid monolithic column. When the concentrations of salts increased, both charges of the monolithic column and DNA were "screened" by the high concentrations of salts. Coulombic forces between
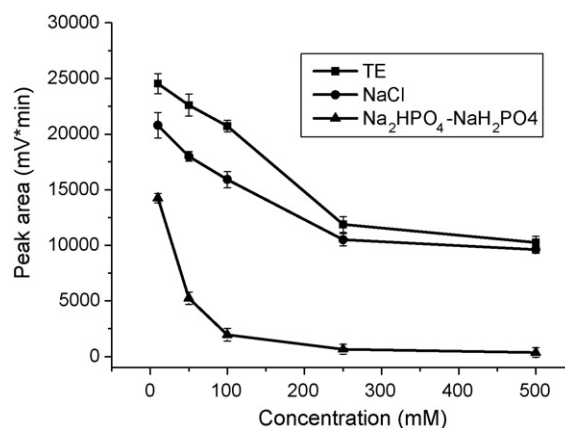

Fig. 6 – Effect of competitive ions on the adsorption behavior of herring sperm DNA. Load condition: 2 ng μL⁻¹ herring sperm DNA in different types of solutions with different concentrations, pH 7.0. Wash condition: 10 mM TE buffer, pH 7.0; elution condition: 200 mM sodium phosphate solution, pH 10.0; flow rate: 240 μL h⁻¹.

the different charged molecules were less involved, and the binding amount of DNA on the monolithic column decreased. The elution peak areas consequently decreased as the concentrations of salts increased. Because competition occurred between phosphate groups of DNA and the phosphate anions from the sodium phosphate solution, the adsorption behavior of DNA could be dramatically influenced by the sodium phosphate solution. While chloride ions are anions, they can adsorb on the positively charged monolithic column. Competition between the chloride ions and phosphate groups of DNA is non-specific. Thus, changes in the elution peak area were not significant for TE or NaCl solution. As high concentrations of salts in the loading solution have been advocated to decrease the undesirable binding of proteins and thus to increase the purity of DNA [24], 50 mM TE buffer at pH 7.0 was chosen for DNA loading, without sacrificing the loading capacity of the monolithic column in the following DNA extraction procedures.

3.3. Effect of sodium phosphate concentration on the DNA recovery

As discussed above, the binding ability of DNA in sodium phosphate solution was the poorest. Thus, sodium phosphate solution at pH 10.0 was used as elution solution to yield high recovery of DNA. The effect of different concentration of sodium phosphate on the DNA recovery under the same loading conditions is shown in Fig. 7. The elution peak areas in 10, 50, 100, 200 and 500 mM sodium phosphate solution were 0, 0, 8625 ± 222, 22637 ± 498 and 25303 ± 639 mV min, respectively. These results suggested that higher concentrations of sodium phosphate could enhance the recovery of DNA. However, high concentrations of sodium phosphate were not beneficial for the subsequent genetic analysis. Thus, 200 mM sodium phosphate solution at pH 10.0 was selected as the optimal condition for effective elution. To remove the possible bound DNA on the monolithic column during consecutive extractions, 500 mM
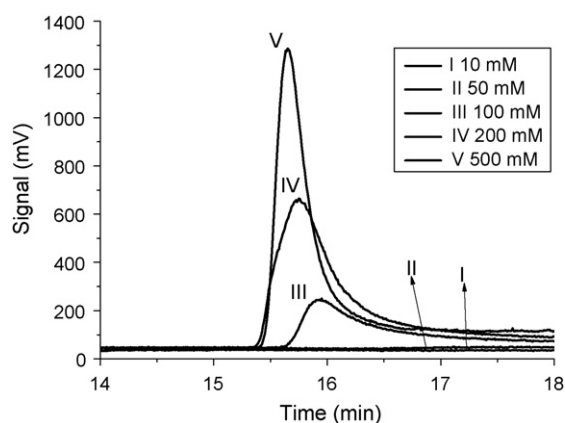
Fig. 7 – The elution profile of herring sperm DNA with different concentration of sodium phosphate solutions at pH 10.0. Load condition: 2 ng μL^{-1} herring sperm DNA in 50 mM TE buffer, pH 7.0. Wash condition: 50 mM TE buffer, pH 7.0; flow rate: 240 μL h^{-1}.

sodium phosphate solution (pH 10.0) and water were applied to wash the column for 5 min each to regenerate the extraction bed.

3.4. Loading capacity of the hybrid monolithic column

The loading capacity of the hybrid monolithic column was investigated by increasing the loading time to increase the amount of DNA under the optimal conditions (load condition: 2 ng μL^{-1} herring sperm DNA in 50 mM TE buffer at pH 7.0. Wash condition: 50 mM TE buffer at pH 7.0. Elution condition: 200 mM sodium phosphate solution at pH 10.0. Flow rate: 240 μL h^{-1}). The elution peak area of DNA increased with the increased loading time when the loading time was less than 6 min (Fig. 8). A plateau was reached after the loading time increased to 6 min. This result implied that the binding amount of DNA on the hybrid monolithic column was almost saturated, corresponding to 48 ng DNA. The high load-
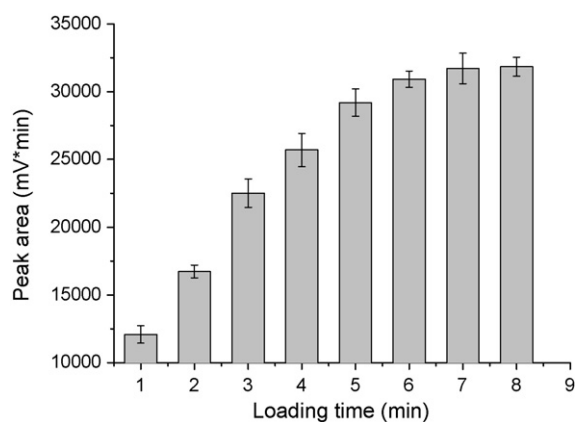
Fig. 8 – Loading capacity of the monolithic column. Load condition: 2 ng μL^{-1} herring sperm DNA in 50 mM TE buffer, pH 7.0. Wash condition: 50 mM TE buffer, pH 7.0; elution condition: 200 mM sodium phosphate solution, pH 10.0; flow rate: 240 μL h^{-1}.
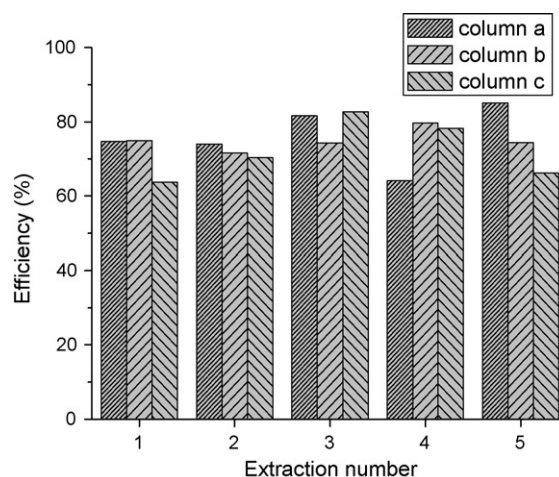
Fig. 9 – Extraction efficiency for five consecutive extractions on three monolithic columns.

ing capacity of this monolithic column is important for its application in real biological samples, especially for the rare samples with small volumes, such as clinical and therapeutic samples.

3.5. DNA extraction efficiency and reproducibility

The extraction efficiency and reproducibility of the prepared monolithic column was assessed by five consecutive extractions on three monolithic columns (Fig. 9). Herring sperm DNA (20 ng) was dissolved in 10 μL TE buffer (50 mM, pH 7.0) as the test samples and 10 μL eluted fraction (in 200 mM sodium phosphate solution at pH 10.0) were collected for DNA quantifications at a flow rate of 240 μL h^{-1}. The extraction efficiency for the three monolithic columns was 74 ± 6.3% for each consecutive extraction. The reproducibility over five consecutive extractions was 6.1%. These results revealed that the prepared hybrid monolithic column could provide high extraction efficiency and good reproducibility, implying that DNA extraction in this miniaturized format was feasible.

3.6. Extraction of the PBE2 plasmid from B. subtilis crude lysate

B. subtilis is able to activate nearly all of the human immune defense systems, including activation of different specific antibodies (such as IgM, IgG and IgA secretion), fungi and bacterial pathogens [25]. It contains the PBE2 plasmid with a length of 7.8 kbp. To verify the prepared monolithic column could extract the target DNA from real samples, the potential of this monolithic column to isolate the PBE2 plasmid from the B. subtilis crude lysate was assessed.

Unlike the extraction of purified DNA, some species in the B. subtilis culture medium, such as protein, RNA, lipids and other inorganic ions, may also adsorb on the monolithic column and influence the purity of the DNA. In addition, the RNA and protein released from B. subtilis cells may also compete with DNA to bind the active sites on the monolithic column. To determine whether this DNA purification protocol could iso-
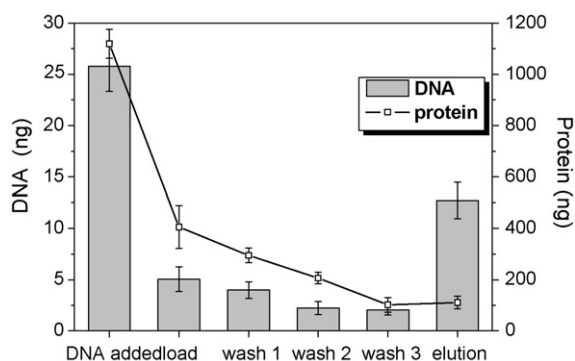
Fig. 10 – DNA and protein extraction profile during the purification of *B. subtilis* crude lysate. The volume of each fraction was 10 μL. Three extractions were performed to evaluate the DNA and protein distribution separately on the monolithic column.

late the target DNA with low contaminants, the amounts of DNA and protein in the extraction process were studied. The *B. subtilis* crude lysate was diluted 20 times in 50 mM TE buffer (pH 7.0) prior to extraction. The optimal procedure developed above was used for the DNA purification: 10 μL samples were loaded onto the monolithic column at a flow rate of 240 μL h^{-1}. The fractions in the load, wash, and elution steps were collected every 10 μL for DNA and protein quantifications. The protein distributions in Fig. 10 suggests that most of the protein (92%) presented in the *B. subtilis* lysate was eliminated during the load and wash steps. Only 110 ng protein was found in the eluted fraction, less than 9% of the original amount in the *B. subtilis* crude lysate. The amount of extracted DNA from the *B. subtilis* crude lysate was about 12 ng, and the extraction efficiency was calculated to be 49%. The extraction efficiency for the PBE2 plasmid was lower than that for purified herring sperm DNA. This appeared to be the result of contaminating species presented in the *B. subtilis* crude lysate. The recovery efficiency of DNA (49%) was much higher than that of protein (9%). This indicated that DNA was preferentially adsorbed on the amino silica column versus protein and that the recovery of DNA from the complex sample was selective. Because DNA was a highly negatively charged molecule, the release of captured DNA with sodium phosphate solution was also selective [26].

The PCR performance of the purified PBE2 plasmid from the *B. subtilis* crude lysate was then identified by CE to indicate the successful extraction of DNA. Fig. 11a shows the DL 2000 DNA marker and Fig. 11b is the positive control in which the PBE2 plasmid extracted by a DNA extraction kit was added directly to amplify the 1600 bp target fragment. Fig. 11c is the negative control in which no DNA template was added to the PCR mixture. Fig. 11d reveals that the DNA extracted by the monolithic column was sufficiently pure for the subsequent PCR amplification. The PCR performance of DNA purified by the monolithic column was similar with that of DNA purified by the DNA extraction kit. Direct PCR of the crude lysate was not successful (Fig. 11e), indicating that the presence of the extraneous cellular and nuclear matter in the crude lysate hampered the DNA amplification. The amplification
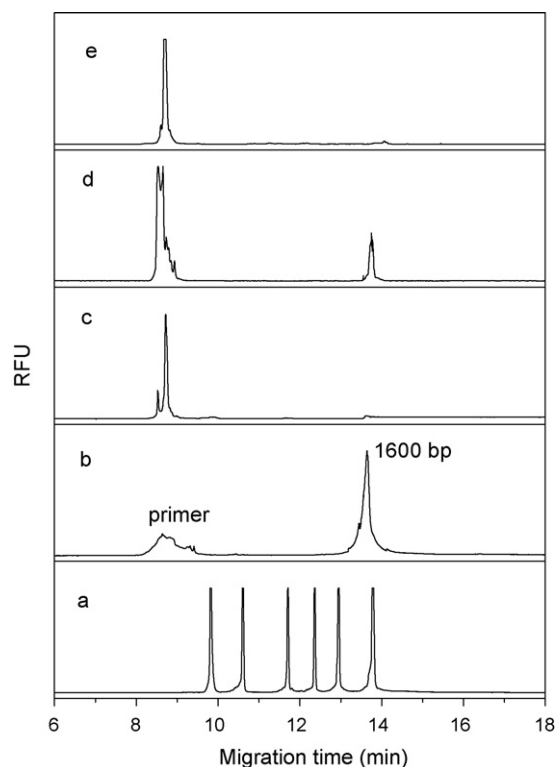
Fig. 11 – Electropherogram of PCR products separated by capillary electrophoresis in 0.20% HEC TME buffer. Electrophoresis condition: −10.00 kV, hydro-dynamic injection for 8 s with 38 cm height: (a) DL 2000 DNA marker, (b) positive control. 1 μL purified PBE2 plasmid extracted by the DNA extraction kit was added directly to the PCR mixture for the PCR reaction, (c) negative control. No DNA was added to the PCR mixture for the PCR reaction, (d) 1 μL purified PBE2 plasmid extracted by the monolithic column was added to the PCR mixture for the PCR reaction and (e) 1 μL *B. subtilis* crude lysate was added to the PCR mixture for the PCR reaction.

performance of the PBE2 plasmid purified from the *B. subtilis* crude lysate illustrated that the prepared monolithic column was suitable for DNA purification from complex biological samples. Because the eluted fraction was free of high concentrations of salts and organic solvents, many PCR inhibitors could be avoided, making the method more appropriate for downstream sample processing. Compared with the commercial DNA extraction kit for DNA purification, the monolithic column required much smaller volumes of DNA samples. In addition, the extraction time was also shorter; less than 25 min was required for the extraction procedure.

4. Conclusions

A new solid-phase support for efficient DNA extraction was developed using the amino silica hybrid monolithic column. The extraction procedure was based on the Coulombic force between DNA and the monolithic column. DNA extraction conditions, such as pH, ion concentration and type, and

loading capacity, were optimized online by CE with LIF detection. Under the optimal conditions, the monolithic column could provide a high extraction efficiency and excellent reproducibility. Because the extraction procedure was carried out in aqueous system without the use of inhibitors, such as high concentrations of salts or organic solvents, the purified DNA was suitable for the subsequent genetic analyses. The PCR performance of the PBE2 plasmid purified from the *B. subtilis* crude lysate demonstrated that the prepared monolithic column was a potential DNA extraction support for DNA purification in miniaturization.

Acknowledgements

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