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New hydroxyapatite monolithic column for DNA extraction and its application in the purification of *Bacillus subtilis* crude lysate

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Abstract

A hydroxyapatite (Hap) monolithic column with micrometer macropores skeleton structure was prepared by sol–gel technique for efficient DNA extraction. The main extraction mechanism of this monolithic column was attributed to the electrostatic interaction between the phosphate groups of DNA and the calcium ions (C site) of Hap. DNA extraction conditions, such as pH, ion concentration, ion type and loading capacity, on the monolithic column were optimized online by capillary electrophoresis with laser-induced fluorescence detection. Under the optimal condition, a 6 cm length monolithic column provided a capacity of 40 ng DNA with an extraction efficiency of $64 \pm 6.2\%$ ($X \pm$ RSD). As low concentration of salts were used in the extraction procedure, the purified PBE2 plasmid from the *Bacillus subtilis* crude lysate could be amplified by polymerase chain reaction. This result illustrated that Hap was a potential matrix for DNA purification from complex biological samples which was compatible with the subsequent genetic analysis in miniature format. Since the preparation of this monolithic column was very simple, it was possible to integrate this novel matrix with chip to allow rapid and efficient DNA purification in microscale. This study provided a new attractive solid-phase support for DNA extraction to meet the miniaturized and automated trends of genetic analysis.

Keywords: DNA extraction; DNA purification; Solid-phase extraction; Hydroxyapatite; *Bacillus subtilis*

1. Introduction

The isolation and purification of DNA from complex biological samples is generally the most important and undervalued step in many biological and biomedical applications. In order to process the subsequent procedures, such as polymerase chain reaction (PCR), DNA cloning, DNA sequencing, DNA hybridization etc., many interfering species (including protein, RNA, and endotoxin) have to be removed [1]. With the development of clinical and forensic applications, the preconcentration and handling of low volume DNA samples is generally required. While the traditional DNA purification methods such as organic extraction and chelex extraction have demonstrated the good ability to remove the interfering contaminants, they are not amenable to the miniaturized and automated trends of genetic analysis because most of these purification procedures require a large amount of DNA samples, reagent volumes, multiple steps and lengthy time [2]. Solid-phase extraction (SPE), which allows both the purification and preconcentration of DNA in one step, while on the other hand, provides an attractive alternative in terms of smaller sample volume requirement, fewer process steps, shorter analysis time, easier miniaturization and automation [3]. In most of the DNA SPE procedures, the DNA molecules can selectively bind to the solid-phase support via electrostatic force or (and) hydrogen bonding force under a particular condition. The unbinded contaminant components can then be washed by the washing buffer. The purified DNA can be subsequently eluted in an aqueous buffer and concentrated into a very small volume. It can be seen that the solid-phase support for DNA adsorption in this process plays a major role as it determines both the dynamic binding capacity and selectivity of DNA.

Contrary to the wide application of solid-phase supports for DNA purification in large quantity [4,5], the supports that could be applied in the miniature format for DNA extraction were

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relatively fewer, which obviously hampered the development of genetic application in miniaturization. This situation may arise from the difficulty in the selection of high capacity solid-phase supports that were suitable to be immobilized in the microchannel of chip and capillary. Several attempts had been tried to address these problems. The most widely used supports for DNA SPE were the silica-based particles. The DNA purification process was mainly based on the selective adsorption of DNA on these particles under high ionic strength chaotropic conditions [6]. Christel et al. used a pillar structure in the microchip to constrain the silica particles for DNA adsorption. While this was an elegant approach for the immobilization of the silica particles, it needed additional fabrication step to create the special structure, which restricted its potential in DNA isolation [7]. Tian et al. developed a capillary-based DNA SPE system by retaining the silica particles in the capillary and demonstrated its potential in DNA purification. However, two glass fiber frits were required to eliminate the loss of silica particles [8]. The problems resulting from the requirement of frits or pillar structure to immobilize the silica particles were in a sense alleviated by the sol–gel technique [9,10]. The sol–gel process generally involved the transformation of sol from liquid state to the solid-phase gel. Thus, the desired structure could be obtained without frits and special microchip fabrication design. The silica particles could be easily immobilized in the microchannel via silica monomers by changing the condition (such as pH or temperature) of the sol containing the silica particles. The *in situ* preparation of silica monolithic phase for DNA SPE in microchannel had proved to be an attractive method for DNA purification [11–13]. However, the shrinkage of the monolithic phase in microchannel had to be controlled, especially for the large diameter capillary (microchip) as the effective surface decreased with the increase in microchannel diameter [14]. Since high concentration of chaotropic salts usually had to be used in the extraction procedures to enhance the interaction between DNA and the silica supports, the chaotropic ions remaining in elution buffer may have dramatic effect on the subsequent genetic process (e.g. PCR analysis) although most of these ions had been removed in the wash step [15]. DNA purification and separation was also carried out on methacrylate-based monoliths [16], such as Convective Interaction Media monolithic columns [17–19]. High concentrations of salts were required to elute the binded DNA from the anion exchange supports effectively. Thus, an additional desalting step was usually needed to decrease the adverse effect of high concentration of salts, especially for the DNA separation by capillary (microchip) electrophoresis and salt sensitive PCR analysis. The ideal solid-phase supports that were amenable to the DNA SPE in miniature format would be the ones that not only provided high productive DNA, but also produced low concentration of salts and inhibitors to be well suited for the subsequent genetic analysis in the extraction procedure.

Several other solid-phase supports had been tested to avoid the use of high concentration of salts and inhibitors in the DNA purification process. Nakagawa et al. [20] used amino silane, which was coated on the open channel, as the solid-phase support for DNA extraction. Since the adsorption of DNA was mainly based on the electrostatic forces between DNA and coated open channel, DNA purification could be easily achieved by changing the buffer from acid range to base range. With a similar approach, Cao et al. [21] coated chitosan on 64 parallel channels in the microchip for effective DNA capture and release in totally aqueous system. The photoactivated polycarbonate microchip also showed its potential in the purification and concentration of genomic DNA from whole cell lysates as demonstrated by Witek et al. [22]. These supports were attractive in their potential compatibility with the subsequent genetic analysis as the inhibitors (e.g. high concentration of chaotropic salts) had been avoided in the extraction procedure. However, special microchip designs were required to enhance the effective surface area for DNA adsorption. The fabrication cost increased consequently, which was restricted in many laboratories.

In this paper, a new hydroxyapatite (Hap, $Ca_{10}(PO_4)_6(OH)_2$) monolithic column was developed for effective DNA extraction under a mild condition. Hap was a ceramic compound with composition simulating the mammalian bone and dentin mineral compartment [23]. It was one of the most favorable biological purification materials due to its excellent biocompatibility and high binding ability for a variety of biomolecules [24,25]. Hap comprised functional groups of positively charged pairs of calcium ions and clusters of negatively charged phosphate groups. Hap had been used in large columns to purify DNA under a few centimeters of hydrostatic pressure to avoid packing of crystals, or in spun columns [24,26]. However, its immobilization in the micrometer range capillary without frits was relatively difficult because it lacked the reactive site to interact with the silanol groups of the capillary. To the best of our knowledge, there was no report on such solid-phase support that was prepared in miniature format for DNA extraction. In the present work, Hap monolithic column for DNA SPE was prepared via sol–gel technique. Hap monolithic column showed its characteristic of micrometer pores that provided low resistance to solution flow for DNA extraction. Extraction conditions, such as pH, ion concentration and ion type, were optimized online for effective DNA purification. The potential application of this Hap monolithic column in the purification of DNA from complex samples was tested by its ability to produce the PCR amplifiable PBE2 plasmid from the *Bacillus subtilis* crude lysate.

2. Experimental

2.1. Chemicals and materials

Sybr green I was obtained from Molecular Probes (Eugene, OR, USA). Tetramethylorthosilicate (TMOS) was purchased from Wuhan University, Silicone New Material (Wuhan, China). Hap particles with 5–10 μm diameter were provided by Biomedical Materials and Engineering Center of Wuhan University of Technology (Wuhan, China). Chitosan ($\eta < 100$ cP, deacylation degree >90%) was bought from Shanghai Bio. Life Science & Technology (Shanghai, China). Tris(hydroxylmethyl)aminomethane (Tris), ethanol, isopropanol, dipotassium hydrogenphosphate (K_2HPO_4) and potassium dihydrogenphosphate (KH_2PO_4) were analytical grade from Shanghai Reagents (Shanghai, China). PCR buffer,

deoxynucleotide triphosphates (dNTPs), Taq polymerase and DL 2000 DNA marker were from TaKaRa Biotech. (Dalian, China). PCR primers were bought from Shanghai Boya Biotechnology (Shanghai, China). Herring Sperm DNA was purchased from Sigma (St Louis, MO, USA). Bicinchoninic acid (BCA) protein assay kit was obtained from Beyotime Inst. Biotech. (Haimen, China).

2.2. Equipment

The monolithic performance of the Hap monolithic column was observed by Olympus BX41 microscope (Olympus Optical, Tokyo, Japan). The cross section of the monolithic column was examined by Quanta 200 scanning electron microscopy (SEM) system (Philips-FEI, Eindhoven, the Netherlands). Prior to SEM imaging, the cross sections containing the immobilized Hap particles were sputter coated with gold for 10 min. PCR was performed in a PTC-100 Peltier Thermocycler (Bio-Rad, Hercules, CA, USA). DNA extraction performance and DNA separation were carried out on the laboratory-built capillary electrophoresis (CE) system with laser-induced fluorescence (LIF) detection [27]. 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 in a personal computer. A 42 cm length (35 cm to the detector) uncoated fused silica capillary (Yongnian Optical Fiber Factory, Hebei, China) with 75 μm I.D. and 365 μm O.D. filled with 0.25% poly(*N*-isopropylacrylamide) (PNIPAM) Tris-Mes-EDTA (TME) buffer (pH 6.11, containing 1:10,000 diluted Sybr green I for DNA intercalation) was used for DNA separation [28]. DNA and protein quantifications were carried out in Spectra max M2 multifunctional microplate reader (MDC, Sunnyvale, CA, USA).

2.3. Preparation of *B. subtilis* plasmid DNA

B. subtilis harboring 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) and was harvested by centrifugation (12,000 rpm, 1 min). Cells were resuspended in water to an approximate density of 10^9 cells/ml, and lysed with lysozyme (100 mg/ml). An amount of 500 μl *B. subtilis* cells containing about 5×10^8 cells were pipetted into a vial from the culture medium. The cells were lysed by adding 5 μl lysozyme. After 15 min incubation at room temperature, the mixture was heated to 37 °C for 30 min to ensure complete lysis. This resulted in a cloudy, viscous solution showing lysis.

2.4. Preparation of Hap monolithic column

A fused silica capillary with 250 μm I.D. and 370 μm O.D. was used to prepare the monolithic column. The capillary was rinsed with 1 M NaOH for 12 h, 1 M HCl for 12 h, water for 1 h, ethanol for 1 h, and subsequently dried under a stream of nitrogen for 3 h, respectively. Then the pretreated capillary was stored at 40 °C prior to use.

The sol–gel precursor solution was prepared by mixing 0.12 g Hap particles, 100 μl ethanol, 100 μl acetic acid solution (1% (w/v), pH 5.0) and 200 μl chitosan acetic acid solution (2.5%, w/v) at 0 °C for 10 min, 100 μl TMOS solution was then added to the above solution. The mixture was ultrasonicated for 5 min at 0 °C after stirring for 35 min. The resultant homogeneous solution was then filled into the pretreated capillary and stored at 40 °C to form the monolithic column. Gelation occurred within 1 h and the gel was subsequently aged in the capillary overnight at the same temperature. The monolithic column was rinsed with ethanol and water for 30 min, respectively before use.

2.5. Preparation of SPE device

A 6 cm length monolithic column was used for the DNA extraction in all extraction procedures. A polyethylene sleeve was used to connect the monolithic column and a 10 cm length fused silica capillary (100 μm I.D. and 365 μm O.D.). The flow rate of the extraction buffers was controlled by pumping the solutions via a positive pressure supplied by the nitrogen cylinders with a gas valve.

For the online evaluations of DNA extraction performance, the other end of the monolithic column was also connected to a 7 cm length fused silica capillary (100 μm I.D. and 365 μm O.D.). A 1 cm length detection window was opened in the middle of the capillary by removing the polyimide coating for online monitoring the change of fluorescence. The prepared extraction device was then assembled on the CE instrument to assess the DNA extraction performance.

2.6. DNA extraction procedure

DNA extraction generally involved three steps: load, wash and elution. Before extraction, the SPE device was equilibrated with 200 mM Tris–HCl buffer (pH 7.0) for 10 min. DNA samples diluted in Tris–HCl buffer were loaded onto the column using pressure injections for various injection times. A wash step was subsequently performed using 80% (v/v) 2-propanol in water to remove unbond DNA and contaminants. DNA was then eluted with 100 mM potassium phosphate buffer. During the consecutive extractions, the Hap monolithic columns were washed with 500 mM potassium phosphate buffer (pH 10.0) and water for 5 min, respectively to regenerate the monolithic column. For the online detection of the DNA extraction procedure on CE instrument, Sybr green I with a diluted concentration of 1:10,000 was added to all buffers to indicate the DNA concentration change.

2.7. DNA and protein quantification

The load, wash, and elution solutions were collected and analyzed for DNA and protein concentrations. DNA amount was detected using Sybr green I ($\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm) in multifunctional microplate reader. Protein amount was also

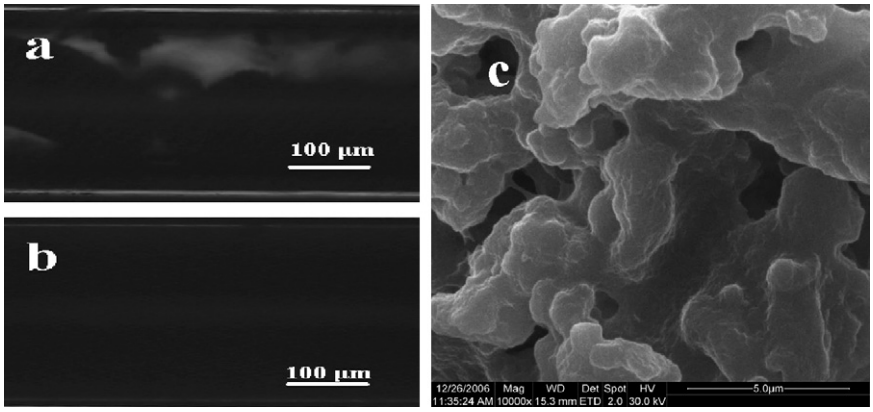

Fig. 1. The morphology of the Hap extraction bed in capillary. (a) Optical microscope image of the Hap extraction bed packed by TMOS sol–gel alone. (b) Optical microscope image of the Hap extraction bed packed by TMOS and chitosan sol–gel. (c) SEM image of the Hap extraction bed in the monolithic column packed by TMOS and chitosan sol–gel.

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), 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 2 μl volume of crude lysate and an equivalent amount of purified PBE2 plasmid extracted by the Hap monolithic column were directly used for PCR amplification.

3. Results and discussion

3.1. Preparation of Hap monolithic column

Hap has been used as a high-performance liquid chromatography column matrix to separate DNA and protein [24,29]. It has also been demonstrated to be a potential vector for gene carrier because of its good biocompatibility [23,25]. While it is an elegant matrix for DNA adsorption, its immobilization in the micrometer capillary is difficult as it lacks the reactive sites to bind the capillary wall. Initial packing of the Hap particles by TMOS sol–gel alone was not successful. As in the optical microscope image illustrated in Fig. 1a, many vacancies with several micrometers were observed along the capillary, indicating that Hap particles were not immobilized equably in the capillary. The addition of 200 μl chitosan acetic acid solution (2.5%, w/v) to the TMOS solution dramatically improved the uniformity of the prepared column. The capillary was evenly packed with Hap particles, and no obvious interstice was observed along the monolithic column (Fig. 1b). The SEM picture of the monolithic column prepared by the TMOS and chitosan sol–gel revealed that the Hap particles had distributed evenly in the continuous bed and formed a skeleton structure with micrometer macropores to allow the extraction solutions to pump easily with low resistance (Fig. 1c). The enhanced stability of the extraction bed by the addition of chitosan to TMOS solution may be ascribed to the three-dimensional (3D) network structure that was formed by the reaction between TMOS and chitosan, which had been shown to encapsulate bovine serum albumin protein for capillary electrochromatography by Kato et al. [30]. In addition, the 3D network structure may also enhance the mechanical stability of the Hap bed, similar to the bonding interaction between Hap particles and the organic matrix prepared by the crosslinkage of chitosan with glutaraldehyde [31]. The stability of the prepared monolithic column was confirmed by 10 continuous DNA extractions without any shatter of the Hap particles during the extraction procedures with different extraction solutions.

3.2. DNA extraction performance on Hap monolithic column

In order to develop an efficient and rapid DNA extraction procedure for DNA purification, some experimental conditions must be carefully optimized. Since the most critical component of the extraction process was the initial adsorption of DNA on the solid-phase support, much attention was paid to investigate

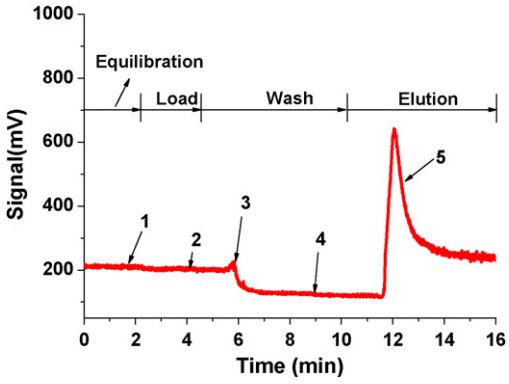

Fig. 2. Typical extraction profile of herring sperm DNA stained with Sybr green I on the Hap monolithic column. (1) Equilibrium baseline (10 mM Tris–HCl buffer, pH 6.0). (2) Loading step baseline (2 ng/μl herring sperm DNA in 10 mM Tris–HCl buffer, pH 6.0). (3) System peak. (4) Washing step baseline (80% (w/v) isopropanol). (5) Elution peak (200 mM potassium phosphate solution, pH 9.0). Running pressure: 6 psi. Flow rate: 300 μl/h.

the factors that influenced the DNA adsorption. As the CE instrument with LIF detection could provide an excellent platform to evaluate the DNA extraction profile online in terms of its high sensitivity and simplicity [12], many experiments were carried out on this platform to assess the DNA adsorption behaviors. Fig. 2 was a typical extraction profile of herring sperm DNA stained with Sybr green I on the monolithic column. Parts 1, 2 and 4 of the profile in Fig. 2 were the baselines of different solutions with different optical properties used in the equilibrium, load and wash steps, respectively. Part 3 of the profile was the system peak that appeared in all extraction procedures. Part 5 of the profile was the elution peak of DNA that adsorbed on the monolithic column. As the adsorption behaviors of DNA under different conditions could be evaluated by calculating the area of the elution peak, DNA extraction conditions, such as pH, ion concentration, ion type and loading capacity, on the monolithic column were optimized by this equipment.

3.2.1. *pH effect of loading solution on DNA adsorption behavior*

Fig. 3b showed that the pH of loading solution had a dramatical effect on the DNA adsorption behavior on the Hap bed.

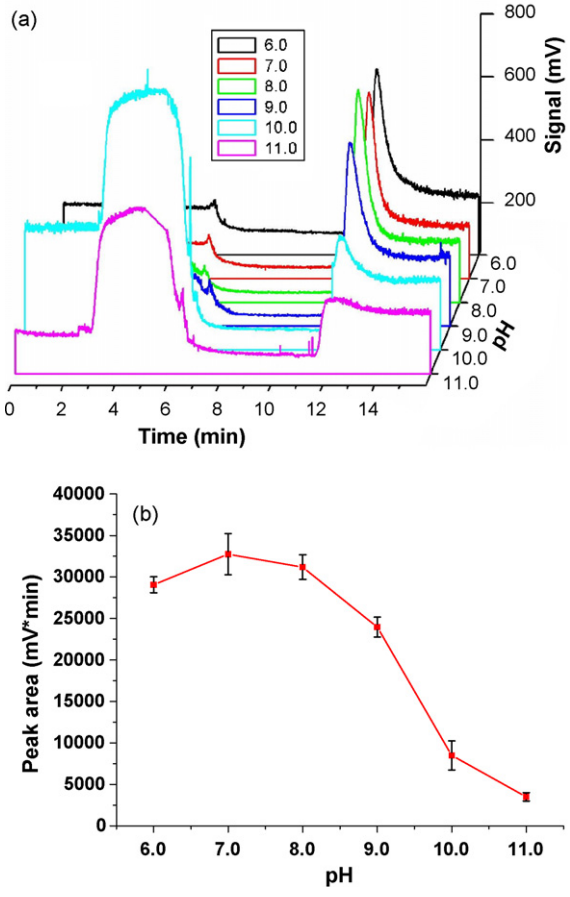

Fig. 3. Effect of pH on the adsorption behavior of herring sperm DNA on the Hap monolithic column. (a) Extraction profile of herring sperm DNA using loading solutions with different pH. (b) pH effect on the elution peak area of herring sperm DNA. Load condition: 2 ng/μl herring sperm DNA in 10 mM Tris–HCl solution with different pH. Other conditions as in Fig. 2.

The elution peak area of DNA in the loading pH ranging from 6.0 to 8.0 had little change, which correlated well with the baseline changes observed in the load and wash steps within the pH range (Fig. 3 a). This result indicated that most of the loaded DNA had been adsorbed on the monolithic column in this pH range. A sharp decrease of the elution peak area was observed when the pH of loading solution raised above 8.0. The elution peak area at pH 11.0 almost decreased to one eighth of that at pH 7.0, which demonstrated that a large percent of the loaded DNA was lost in the load and wash steps at this pH (Fig. 3a). Zhu et al. [23] had shown that Hap particles could bind DNA under acidic and neutral conditions, but could not under alkaline condition. Our results were in agreement with this report. The zeta potential of Hap nanoparticles revealed that the particles were positively charged in the range from 6.0 to 9.5 and were negatively charged under more alkaline condition [25]. Since the anionic property of DNA in the above solution could not change significantly, the different retention behaviors of the DNA might arise from the different charged status induced by pH change of the loading solution. The Hap monolithic column was positively charged under low pH condition (pH < 9.0) to allow the adsorption of the reverse charged DNA and was negatively charged under high pH condition (pH > 9.0) to repel the same charged DNA. The understanding of pH dependence of DNA adsorption on the Hap monolithic column would be very important for DNA extraction process. The DNA extraction thus could be easily realized by changing the pH of the solutions for efficient DNA adsorption and desorption. As the neutral condition could avoid the precipitation of protein and lipid in biological samples, pH 7.0 was chosen as the optimal condition for the loading solution. From the above results, it could also be concluded that an increase of pH would make DNA elution much easier. Thus, pH 10.0 was selected as the optimal elution condition without further optimization.

3.2.2. *Effect of competitive ions on DNA retention behavior*

pH 7.0 was chosen as the optimal loading pH to investigate the effect of different types of competitive ions with different concentrations on the DNA adsorption behavior. As shown in Fig. 4, the elution peak area in all the loading solutions of Tris–HCl, KCl (dissolved in 2 mM Tris–HCl buffer at pH 7.0) and KH_2PO_4–K_2HPO_4 decreased with the increase in salt concentrations. However, among the ions presented in the solutions ($Tris^+$, K^+, Cl^-, and phosphate), the phosphate anion had the most significant effect on the adsorption of DNA on the monolithic column. Only 8.3% of the elution peak area was observed in 500 mM KH_2PO_4–K_2HPO_4 buffer as compared with that in 10 mM buffer. For the Tris–HCl loading buffer, the change of elution peak area was not obvious, and the lowest elution peak area in 500 mM buffer was 92.5% of the highest one in 10 mM buffer. For the KCl solution, the ratio of the lowest elution peak area versus the highest one was 70.7%. As different types of ions with the same concentration were concerned, the KH_2PO_4–K_2HPO_4 loading buffer had the most powerful competitive ability, while the Tris–HCl buffer the lowest. These results could be explained by the adsorption mechanism of DNA on Hap crystals [32,33]. All the bases of DNA molecules were on

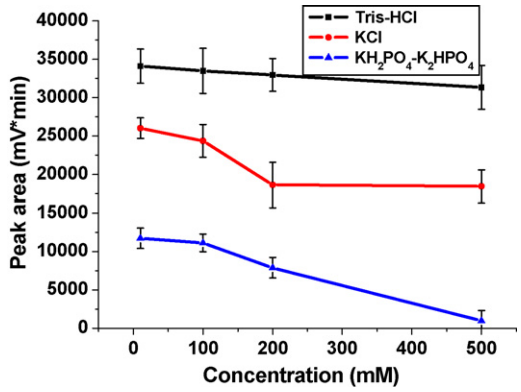

Fig. 4. Effect of competitive ions on the adsorption behavior of herring sperm DNA. Load condition: 2 ng/μl herring sperm DNA in different types of solutions with different concentrations, pH 7.0. Wash condition: 80% (w/v) isopropanol. Elution condition: 200 mM potassium phosphate solution, pH 10.0. Running pressure: 6 psi. Flow rate: 300 μl/h.

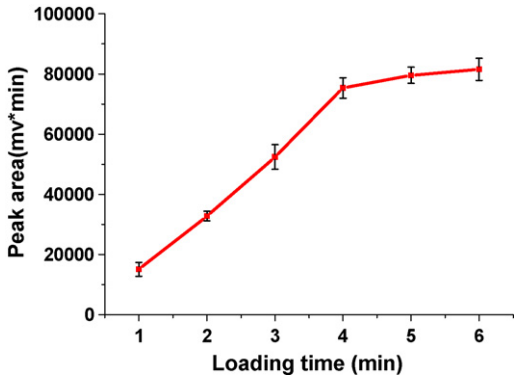

Fig. 5. Loading capacity of the Hap monolithic column. Load condition: 2 ng/μl herring sperm DNA in 200 mM Tris–HCl buffer with pH 7.0. Other conditions as in Fig. 4.

the inside of the double helix, with the sugar phosphates on the outside. Since the phosphate groups were negatively charged, it could be speculated that the DNA would bind to Hap because of the strong affinity between the phosphate groups and the calcium ions (C site) of Hap. However, phosphate ions from the KH_2PO_4–K_2HPO_4 loading buffer were also absorbable onto the C sites although the potassium ions from the same buffer were only absorbable onto P sites of Hap. Therefore, competitions occurred between DNA and phosphate ions on C site in the potassium phosphate system. Thus, the KH_2PO_4–K_2HPO_4 loading buffer had a dramatical effect on the DNA adsorption behaviors. While chloride ions were also anions, they were virtually unabsorbable on the Hap surface, presumably owing to the steric hindrance. That was why the change of elution peak area was not significant for the Tris–HCl and KCl loading solutions. It was reported that monovalent cations could interact with Hap surface to increase the DNA binding affinity [34,35]. For the $Tris^+$ and K^+ presented in the loading solutions with the same concentration, $Tris^+$ might interact more firmly than that of K^+ in a way similar to the interaction between the basic protein and Hap [36]. Therefore, the influence of K^+ on the binding behavior of DNA was much larger than that of $Tris^+$. This binding effect resulted in the elution peak area of DNA in Tris–HCl buffer was to be higher than that in KCl solution. As high concentration of salts in the loading buffer during DNA loading was normally advocated to decrease the binding of undesirable proteins and thus to increase the purity of DNA [37,38], 200 mM Tris–HCl with pH 7.0 was chosen to load DNA in the following DNA extractions.

3.2.3. Loading capacity of the monolithic column

The loading capacity of the monolithic column was investigated by increasing the loading time to calculate the DNA amount under the optimal loading condition. As shown in Fig. 5, the elution peak area increased with the increase in loading time, when the loading time was less than 4 min. A plateau reached after the loading time increased to 4 min. This result indicated that the binding amount of DNA on the monolithic column was almost saturated, which corresponded to 40 ng DNA. The DNA capacity of the monolithic bed was calculated to be 4.2 μg/g. The high binding capacity of the monolithic column would be very important for its application in real biological samples, especially for those of rare DNA sources in clinical and therapeutic applications.

3.2.4. Effect of potassium phosphate concentration on recovery

As discussed above in the adsorption behavior of DNA in different types of solutions, the binding ability of DNA in the potassium phosphate solution was the poorest. Thus, potassium phosphate solution at pH 10.0 was used as the elution solution to yield high recovery of DNA on the monolithic column. Different concentrations ranging from 10 to 500 mM were tested to elute the DNA under the same loading condition. As illustrated in Fig. 6, the elution peak area of DNA increased with the increase in potassium phosphate concentration. Five hundred millimolar potassium phosphate solution was found to yield the highest DNA elution peak area. The elution peak area of DNA did not increase significantly when the concentration of potassium phosphate increased further. A backpressure increase was observed when the concentration of potassium phosphate increased higher. Although high concentration of potassium phosphate may enhance the DNA recovery, too higher concentration of salts would not fit for the subsequent genetic analysis and electrophoretic separation. As a compromise between the recovery and the convenient process for the subsequent DNA analysis, 100 mM potassium phosphate at pH 10.0 was selected as the optimal concentration for efficient DNA elution. During the consecutive extractions, 500 mM potassium phosphate buffer (pH 10.0) was chosen to wash the extracted columns for 5 min to avoid the possible binded DNA on the monolithic column. The monolithic column was subsequently washed with water for 5 min to elute the potassium phosphate solution.

3.3. DNA extraction efficiency and reproducibility

After all the extraction parameters have been optimized, the extraction efficiency and reproducibility of the prepared mono-

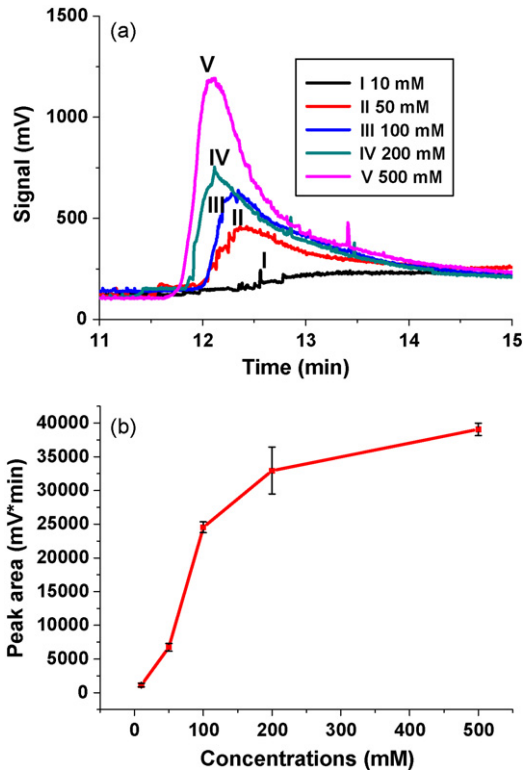

Fig. 6. Effect of potassium phosphate concentrations on DNA elution ability. (a) Elution profile of herring sperm DNA with different concentrations of potassium phosphate. (b) Effect of different concentrations of potassium phosphate on the elution peak area of DNA. Elution condition: different concentrations of potassium phosphate solution at pH 10.0. Other conditions as in Fig. 4.

lithic column was examined by five consecutive extractions on three monolithic columns (Fig. 7). The average extraction efficiency of the three monolithic columns was 64% with a RSD of 6.2%. The RSD of different columns was 6.1%. These results indicated that Hap monolithic column prepared by the sol–gel technology provided high extraction efficiency and high reproducibility, which implied that DNA extraction in microscale format in this column was feasible.

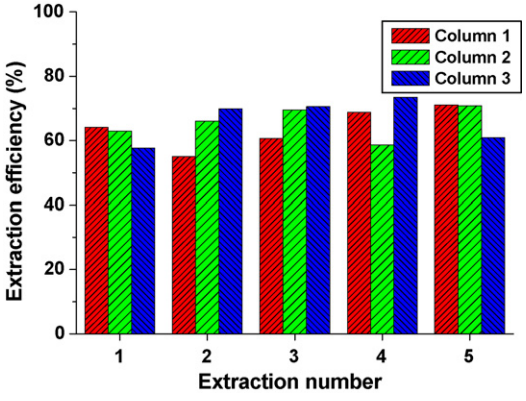

Fig. 7. Extraction efficiency for five consecutive extractions on three Hap monolithic columns. Elution condition: 100 mM potassium phosphate solution at pH 10.0. The Hap monolithic columns were washed with 500 mM potassium phosphate solution (pH 10.0) for 5 min and water for 5 min, respectively during the consecutive injections. Other conditions as in Fig. 4.

3.4. Extraction of PBE2 plasmid from B. subtilis crude lysate

B. subtilis which contains PBE2 plasmid (7.8 kbp) is a Gram-positive, rod-shaped and endospore-forming aerobic bacterium. It is found in soil and rotting plant material and is non-pathogenic. *B. subtilis* has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. Unlike several other well-known species, *B. subtilis* has historically been classified as an obligate aerobe, though recent research has demonstrated that this is not strictly correct [39]. It remains one of the most potent and beneficial of all health-promoting and immune-stimulating bacteria. According to clinical studies documented in the medical research report, the cell wall components of ingested *B. subtilis* are able to activate nearly all systems of the human immune defense, including the activation of at least three specific antibodies (IgM, IgG and IgA secretion) which are highly effective against many of the harmful viruses, fungi and bacterial pathogens which regularly attempt to invade and infect the human system [40]. As a result, the purification of the related gene from this bacterium is a key procedure for its application in clinic and therapeutic.

The usual method applied to purify the PBE2 plasmid from *B. subtilis* consisted of the following steps: cell lysis using lysozyme, RNA removal by RNase, extraction and precipitation with organic solvents and ultracentrifugation in density gradients. Due to their initial design, they were very time consuming and not amenable to the DNA purification in microscale. Other problematic issues included the use of flammable liquids, materials that were not certified for application in humans, enzymes from avian or bovine origin and toxic reagents such as phenol, CsCl or CsBr [18]. To meet the trend of purifying the characteristic gene in automated and miniaturized form, the prepared monolithic column was applied to test the possibility of purifying the PBE2 plasmid directly from the cultured *B. subtilis* crude lysate.

In order to investigate the effect of protein that was presented in the *B. subtilis* lysate on the DNA extraction performance, the amounts of DNA and protein that changed during the extraction process were studied. Since the high viscosity of the LB medium would increase the backpressure of the column, the cultured *B. subtilis* crude lysate was diluted to 30 times by 200 mM Tris–HCl loading buffer for convenient loading prior to extraction. As shown in Fig. 8, only 1.99 and 2.04 ng DNA was lost during the load and wash step, respectively. The extracted DNA from the 10 μl diluted *B. subtilis* lysate was 11.2 ng with a recovery of 61.1%, indicating that most of the binded DNA was obtained in the elution fraction. The extraction efficiency of DNA purified from *B. subtilis* lysate was somewhat lower than that of herring sperm DNA. This appeared to result from the contaminated species that presented in the *B. subtilis* lysate, which may also compete with DNA to bind the adsorption sites. The protein distribution in Fig. 8 suggested that most of the protein (74.8%) presented in the *B. subtilis* lysate was removed by the denatured reagent, isopropanol. This result corresponded well with the reported literature that the denatured protein was unable to bind Hap [41]. The amount of protein in the elution

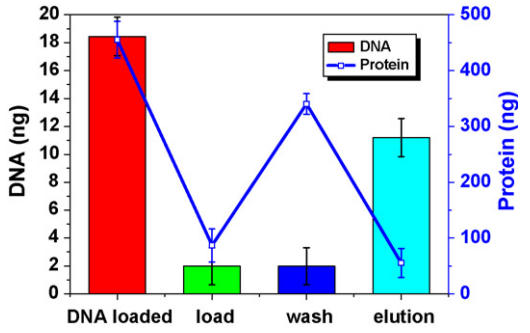

Fig. 8. DNA and protein extraction profile during the purification of *B. subtilis* crude lysate. Load condition: 10 μl diluted *B. subtilis* crude lysate in 200 mM Tris–HCl loading buffer at pH 7.0. Other conditions as in Fig. 7. Three extractions were performed to evaluate the DNA and protein distribution separately on the monolithic column.

buffer was 53.6 ng, which was less than 11.6% of the amount that presented in the *B. subtilis* lysate. From Fig. 8, it could be concluded that the long-term stability of the monolithic column was good since the RSD values of the DNA and protein amount during the extraction procedures were less than 12.1% and 25.6%, respectively. These results demonstrated that the extraction method was successful to purify the DNA with low protein interferences from the complex samples for the subsequent DNA analysis.

Finally, the elution fraction was directly added to the PCR reaction mixture to determine whether the extracted DNA was

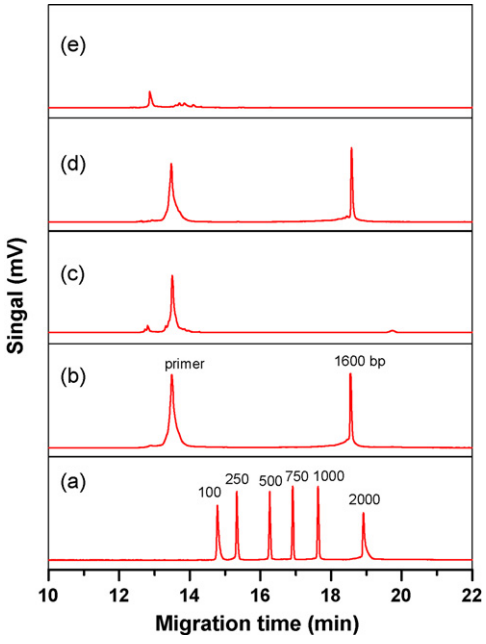

Fig. 9. Electropherogram of PCR products separated by capillary electrophoresis in 0.25% PNIPAM TME buffer. Electrophoresis condition: −8.00 kV, hydrodynamic injection for 10 s with 38 cm height. (a) DL 2000 DNA marker. (b) Positive control. PBE2 plasmid purified by DNA extraction kit was added directly to the PCR mixture for PCR reaction. (c) Negative control. No DNA was added to the PCR mixture. (d) 2 μl purified PBE2 plasmid extracted by the Hap monolithic column was added to the PCR mixture for PCR reaction. (e) 2 μl *B. subtilis* crude lysate was added to the PCR mixture for PCR reaction without purification.

PCR amplifiable. In order to make a comparison, a 2 μl volume of the crude lysate and an equivalent amount of purified PBE2 plasmid extracted by the commercial available DNA extraction kit were also simultaneously subjected to PCR. The PCR products were subsequently identified by CE with LIF detection. Fig. 9a was DNA marker with size ranging from 100 to 2000 bp, and Fig. 9b was the positive control in which the purified PBE2 plasmid extracted by DNA extraction kit was added directly to amplify the 1600 bp fragment. The absence of the target fragment in Fig. 9c was the negative control, in which no DNA was added to the PCR mixture to confirm that no other source of DNA fragment was presented in the PCR procedure to amplify the 1600 bp fragment. Fig. 9d revealed that the DNA extracted by the monolithic column was pure enough for the subsequent PCR amplification. The PCR performance of the eluted DNA was equivalent to that of DNA purified by DNA extraction kit. The direct PCR using the crude lysate was not successful (Fig. 9e), indicating that the presence of the extraneous cellular and nuclear matter in the crude lysate hampered the DNA amplification.

The extraction performance of PBE2 plasmid from *B. subtilis* crude lysate illustrated that the Hap monolithic column was suitable to extract DNA from complex biological samples. Compared with the commercial DNA extraction kit for DNA purification, the sample volume needed to load on the monolithic column was much smaller. In addition, the extraction time was also shorter as less than 20 min were required for the extraction procedure.

4. Conclusion

A Hap monolithic column with micrometer macropores skeleton structure was prepared for efficient DNA extraction. DNA extraction conditions, such as pH, ion concentration, ion type and loading capacity, on the monolithic column were optimized online by CE with LIF detection. The electrostatic interaction between the phosphate groups of DNA and the calcium ions (C site) of Hap was the main driving force for the DNA extraction. Under the optimal condition, the monolithic column could provide excellent reproducibility and high extraction efficiency. The extraction performance of PBE2 plasmid from *B. subtilis* crude lysate demonstrated that the Hap monolithic column was suitable to extract DNA from complex biological samples. As low concentrations of salts were used in the extraction procedure, the extracted DNA was amenable to the subsequent PCR analysis without inhibitors. Since the preparation of this monolithic column was very simple, it was possible to integrate this new solid-phase support with the chip to allow rapid and efficient DNA purification in microscale. This study provided a new attractive solid-phase support for DNA extraction to meet the miniaturized and automated trends of genetic applications.

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