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RESEARCH ARTICLE

CHEN Xing, CUI Dafu, LIU Changchun, LI Hui, ZHAO Weixing

Sample pretreatment microfluidic chip for DNA extraction from rat peripheral blood

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Abstract A sample pretreatment microfluidic chip was described based on the principle of solid phase extraction and micro electro mechanical system technology. Oxidized porous silicon with the large surface area as the solid phase matrix for absorption of DNA from a biological sample can greatly improve the DNA yield. The factors that could affect the DNA yield were analyzed and the preparation technology and the experiment procedure were improved. The DNA purification process from the rat peripheral blood can be achieved and the DNA yield is 24 ng/(μL whole blood), which can reach the level of the commercial DNA purification kits. Furthermore, the DNA extracted from the whole blood can be amplified by polymerase chain reaction, which can achieve a high efficiency of the amplification.

Keywords micro electro mechanical system, oxidized porous silicon, sample pretreatment microfluific chip

Various reactions such as polymerase chain reaction (PCR), electrophoresis, separation, hybridization, etc. in the fields of DNA analytical detections or illness detections need sufficient DNA with high quality. There are considerable researches done on PCR microchip, capillary electrophoresis microchip, and hybridization microchip [1–5]; however, there are few reports about the DNA extraction microfluidic chip. The research on DNA extraction microchip is the key technology for the integrated automation microfluidic system. Solid phase extraction (SPE) is an extraction method for purifying analyte-based substances on the surface characteristics of solid phase and ion-exchange principle. The general procedure of SPE is to load a sample mixture onto the SPE phase for non-selective absorbing analyte on the solid phase matrix, wash away undesired components, and then desorb the desired analyte with the eluted solvent. During the procedure of SPE, DNA can hardly be degraded, and the purity and yield of the extracted DNA can be improved by using the protein denaturant and the reagent for degrading RNA. The surface of silicon, glass, ion-exchange resin, modified magnetic beads, polymer and so on can be usually used as the solid phase matrix for extracting DNA [6–8]. Tian et al. [9] reported a sample pretreatment device for DNA purification, in which silica micro beads were directly packed in a capillary as the solid phase matrix. Wolfe et al. [10] immobilized bare silica beads matrix in the channels of the microchip by sol-gel technology for extracting DNA from blood, bacteria, and virus samples. The advantage of the method of packing matrix is its flexibility to change the solid phase matrix. However, this method has so many disadvantages. A high packing density for larger surface area in the microfluidic devices results in problems with backpressure as well as clogging with crude samples. It is also complex to pack the solid phase matrix, and it is difficult to control small particles in the microfluidic devices. Moreover, this method could not be compatible technology for integrating with other microchips. Cady et al. [11] and Christel et al. [12] reported DNA extraction microchips by using pillars etched in silicon wafers as the solid phase matrix. The design of pillars can increase the surface area available for DNA adsorption. However, the increase of the surface area was limited, which resulted in lower yield of the extracted DNA, and the problems of clogging could not be completely avoided.

In this paper, an oxidized porous silicon microfluidic chip for extracting DNA is designed and prepared. The yield of the extracted DNA can be improved much more due to the large surface area of the oxidized porous silicon matrix. Moreover, the purified DNA was successfully used in PCR reaction.

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CHEN Xing, CUI Dafu (✉), LIU Changchun, LI Hui
State Key Laboratory of Transducer Technology, Institute of Electronics, Chinese Academy of Sciences, Beijing 100080, China
E-mail: dfcui@mail.ie.ac.cn

ZHAO Weixing
State Key Laboratory of Protein and Plant Genetic Engineering, College of Life Sciences, Peking University, Beijing 100871, China

1 Experimental

1.1 Blood sample and reagents

Rat whole blood was collected from the orbital sinus of rat into Vacutaner tubes containing the anticoagulant heparin for

every 10 μL. λ-DNA, Guanidine HCl and Triton-X 100 were purchased from Xinjingke (Beijing, China). SYBR Green I dye was purchased from BioWhittaker Molecular Applications (Rockland. Me, US). PCR kit and DNA extraction kit were purchased from Tianwei (Beijing, China). Primers 5′-AGAAGTACCTGCAACAGG-3′ and 5′-GACGGACACATTGGGGGT-3′ for the 203-bp, -*gapd* gene were purchased from Sangon (Shanghai China). All solutions were prepared in distilled water. TE buffer (10 mmol/L Tris, 1 mmol/L EDTA, titrated to pH 8), load buffer (6 mol/L GuHCl in TE buffer, titrated to pH 6.7, namely A solution), wash buffer (6 mol/L GuHCl in 10 mmol/L Tris, titrated to pH 6.7, namely B solution) were prepared in our laboratory.

1.2 Design and fabrication of microchip

The oxidized porous silicon microfluidic chip is shown in Fig. 1. The size of this chip is 2 cm × 1 cm, the micro channel in the silicon substrate is 200 μm wide, 140 μm deep, and 25 cm long, and the inlet and outlet ports are 2 mm in diameter. The surface of the internal wall of the micro channel is oxidized porous silicon. The process used to fabricate the microchip is as follows. The silicon wafers were coated with silicon nitride, which could be a mask for anisotropical etching and electrochemical etching, after the wafers were cleaned. Then the wafers were spin coated with a negative photoresist (BN303) and patterned. After the exposed photoresist was developed, the exposed silicon nitride on the wafers was removed by plasma etching. Then the wafers were etched in 33% potassium hydroxide (KOH) by weight to produce a micro channel, inlet, and outlet ports. Then a porous silicon layer was obtained on the internal wall of the micro channel by electrochemical etching technology. Then the wafers were thermally oxidized, making porous silicon layer perform oxidized porous silicon layer. Finally, a Corning Pyrex#7740 glass cover was anodically bonded to the silicon wafer to form a close channel by a bonder (Suss, SB6), after the nitride were removed by plasma etching.

1.3 Extraction DNA from whole blood

(1) Microchips were washed with HNO_3 and TE buffer for 5 min prior to each extraction; (2) DNA adsorption: load buffer with 10 μL whole blood and 4 μL Triton X-100 and 100 μL A solution, which were uniformly mixed and then incubated in water bath at 40–55°C for 5 min for lyzing cells, was passed through the chip at a flow rate of 10–15 μL/min for 8–10 min. After the load buffer flowed out from the microchip, the solution was collected and then marked S_1; (3) Impurity washing: washing buffer with 30 μL B solution and 30 μL ethanol was fully mixed and passed through the chip at a flow rate of 20–25 μL/min for 2.5–3 min. After this washing buffer flowed out, this solution was collected and then marked S_2. Then 70% ethanol solution (by volume) was passed through the chip at the flow rate of 20–25 μL/min for another 2.5–3 min. After this washing solution flowed out, every 30 μL solution was collected and then marked S_3 and S_4; (4) DNA elution: 20 μL TE buffer was introduced in the chip and incubated in the chip for 10 min and then flowed out. This TE buffer was collected and marked S_5. Then 100 μL TE buffer was continuously passed through the chip at a flow rate of 5–10 μL/min for 10–20 min. Every 20 μL TE buffer was collected and marked S_6–S_{11} in turn.

1.4 DNA detection

DNA collected in the load, washed and elution solutions were quantified by using SYBR Green I dye tagging DNA. A real-time quantitative PCR detecting system was used to detect the intensity of fluorescence and then the DNA concentration was calculated by using calibration curves which were generated using lambda DNA. 10 μL solutions from S_1–S_{11} were added with 4 μL SYBR Green-I (V:V = 1:1 000) and 11 μL TE buffer, respectively. Then these solutions were marked S'_1–S'_{11} in turn.

The DNA extracted from whole blood was qualified by polymerase chain reaction (PCR) and gel electrophoresis. A 5 μL DNA extracted by microchip was used as the template of the PCR to amplify the 203-bp fragment of *Gapd* gene, in addition with positive and negative controls. The PCR reactions consisted of 2.5 μL of standard 10 × PCR buffer, 100 μmol of dATP, dGTP, dCTP, and dTTP, 2.5 units of Taq polymerase, 50 nmol/L of each primer, and 5 μL of the initial collected fraction, in a total volume of 25 μL. These reactions were cycled under the following conditions: 95°C denaturation for 5 min, 35 cycles of 94°C for 1 min, 68°C for

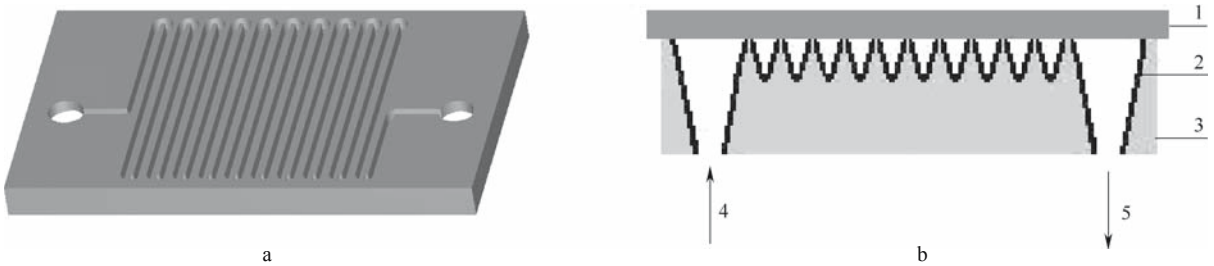


Fig. 1 Layout of the chip
a) Top view of the silicon substrate; b) Cross-section view of the chip.
1) Glass; 2) Oxidized porous silicon; 3) Silicon; 4) Inlet port; 5) Outlet port.

1 min, 72°C for 1 min, followed by a 10 min extension at 72°C. The PCR products were then run through gel electrophoresis.

2 Results and discussion

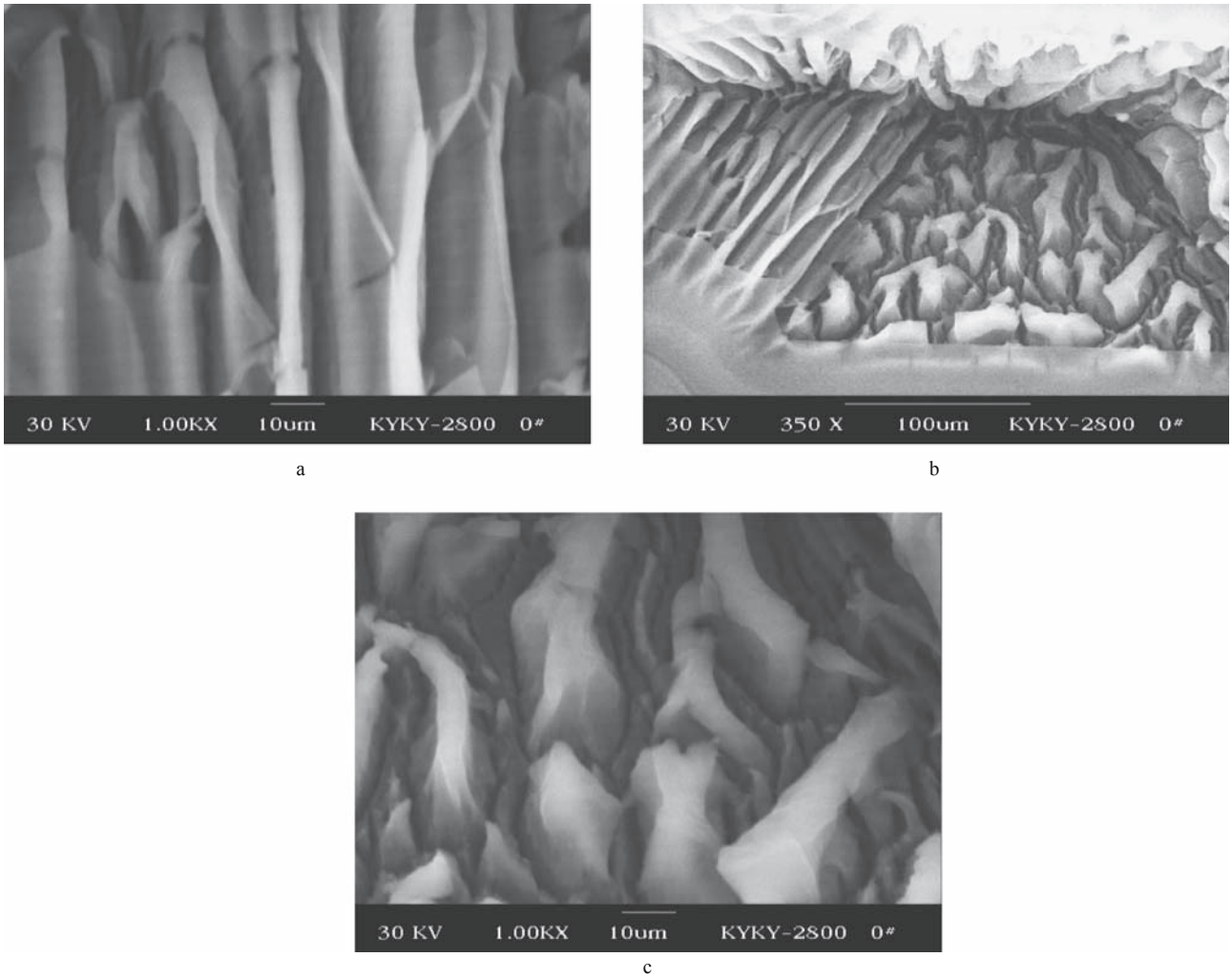
2.1 Effect of conditions of electrochemical etching on morphology of porous silicon

Porous silicon was fabricated by using electrochemical etching technology. The conditions of electrochemical etching are shown in Table 1.

The effect of the conditions of electrochemical etching on the micro structure of the porous silicon was investigated at different etching time, current density and concentration of electrolyte. The results show that the surface morphology of porous silicon is sponge-type and insensitive of etching time, when the concentration of HF and the current density are kept constant. The thickness of the porous silicon layer increases with the etching time. Approximately 30 μm porous silicon layer was obtained for the etching time of 20 min. However, longer etching time resulted to the upper layer of porous silicon peeling off from the bulk silicon. The surface morphology of porous silicon is sensitive of current density, when the concentration of HF and the etching time are fixed. At high current density, the porous silicon is branch-type, for example Protocol B, shown in Fig. 2(a). At low current density, the porous silicon is sponge-type, for example Protocol F, shown in Fig. 2(c). But high current density might lead to

Table 1 Porous silicon fabrication projects by anodation method

Protocol	Etchtime / min	Concentration of solution (HF : ethanol)	Current density / ($mA \cdot cm^{-2}$)
A	5	20:80	62.5
B	20	20:80	62.5
C	60	20:80	62.5
D	20	20:40	62.5
E	20	20:20	62.5
F	20	20:80	25
G	20	20:80	125
H	5	20:20	125



a b c

Fig. 2 SEM images of the porous silicon

surface crack of the silicon wafer. When the current density and the etching time are fixed, it is easy to obtain the sponge-type porous silicon at high concentration of HF, whereas the blending porous silicon of branch-type and sponge-type can be obtained at a middle concentration of HF, for example Protocol D (Fig. 2b). When the concentration of HF and current density are both higher, the silicon wafer is polished and does not form porous silicon, for example Protocol H. Porous silicon with a relatively large specific surface area-to-volume can significantly increase the available interface area for adsorbing DNA after thermal oxidization. In this paper, Protocol B was used to fabricate a microchip for extracting DNA, in which the porous silicon layer is branch-type and 30 μm thick.

2.2 Effect on the yield of DNA extracted by microchip

The DNA purification process employed here utilizes adsorption of DNA onto a solid phase matrix of silicon dioxide or glass under high ionic strength chaotropic conditions and low pH, and then desorption of DNA from the matrix under low ionic strength chaotropic conditions and high pH. The high ionic strength serves to shield the negative surface, reducing the electrostatic repulsion between the negative DNA and the surface of the matrix, while the chaotropic salt dehydrates the matrix surface and DNA, thus promoting hydrogen bonding between the DNA molecules and the protonated silanol groups. The quantity of adsorption DNA is linearly increased with the quantity of silanol groups of solid phase matrix. The yield of extracted DNA is not only affected by the type of salt, concentration, and pH of the solution, but also by the specific surface area-to-volume and surface characteristics of the solid phase matrix. According to the literatures [9–12] and earlier research [10], 6 mol/L GuHCl at pH 6.7 was used as bonding salt for extracting DNA. Non-porous silicon dioxide microchip for extracting DNA was fabricated by means of the literature [13], while porous silicon microchip was fabricated by Protocol B and then oxidized at room temperature and higher temperature to perform the oxidized porous silicon microchip for extracting DNA. Under the same conditions, these two kinds of microchips were used to extract genomic DNA from the whole blood of rat. The yield of extracted DNA was measured by the fluorescence reagent of SYBR Green I. The intensity of fluorescence in the presence of double-stranded DNA is much higher than without double-stranded DNA. Moreover, the intensity of fluorescence is linearly increased with the concentration of DNA. Thus, the concentration of DNA could be calculated by means of a calibration curve of DNA which was generated using lambda DNA. 15.7 ng genomic DNA was extracted from per microlitre whole blood by using non-porous silicon dioxide microchip, while 9.2 ng genomic DNA by using oxidized porous silicon microchip that had been oxidized at room temperature. The yield of extracted DNA was affected by the temperature of oxidization. Approximately 13–15 ng genomic DNA was extracted by using oxidized porous silicon microchip that had been oxidized at or below 400°C, whereas approximately 23–27 ng of genomic DNA was extracted by using oxidized porous silicon microchip that had been oxidized at 500–700°C. But the silicon wafer was heavily distorted during the process of transforming porous silicon to oxidized porous silicon at higher temperature, and then it could not bond to the cover. Therefore, the yield of extracted DNA was zero by using this microchip. Compared to non-porous silicon dioxide, oxidized porous silicon has a huge surface area and much more silanol groups for adsorbing DNA, achieving the improved yield of extracted DNA. When the porous silicon was oxidized at room temperature or low temperature, the oxidized porous silicon layer might be thinner and the quantity of Si–O bonds was small [14], thus the silanol groups were less and the ability of adsorption of DNA was weak. In this paper, the oxidized porous silicon microchip that had oxidized at 500°C was used to extract DNA.

2.3 Extraction DNA from rat peripheral blood

Genomic DNA was extracted by using the oxidized porous silicon microchip that had oxidized at 500°C, and the intensity of fluorescence of all the collected samples was detected by using fluorescence method. The concentration of DNA in all the collected samples was obtained by means of a calibration curve of DNA, which was generated using lambda DNA. The results are shown in Fig. 3. The concentration of DNA in sample S'_1 is 0 ng, which means that the genomic DNA is almost adsorbed onto the oxidized porous silicon during the load step. The concentration of DNA in samples S'_2–S'_4, which represent the collected solution during the washing step, is small. This means that only a little DNA was desorbed during the washing step. Samples S'_5–S'_{11} represent the eluted solutions for desorbing DNA from oxidized porous silicon. From Fig. 3, the value of S'_5 is only 22.4 ng, which represents the eluted DNA desorbed during the first eluted step, and the value of S'_6 is 39.5 ng, the highest point, which represents the

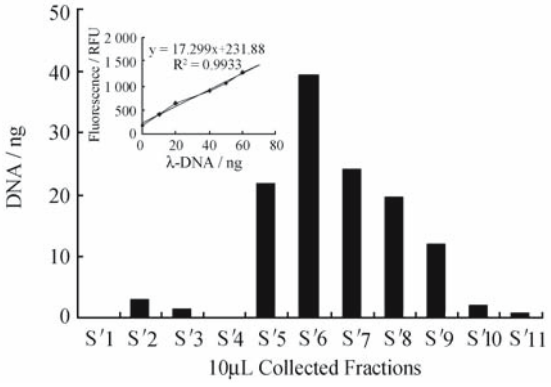


Fig. 3 DNA concentration profile for DNA purification
Loading step: S′1; Washing step: S′2–S′3; Elution step: S′ 5–S′11; Insert: the standard curve for DNA by using λ-DNA

eluted DNA desorbed during the second eluted step. The maximum point of desorbed DNA was not present in the first eluted step, that means the process of DNA desorption from oxidized porous silicon is slow. Overall, 97.6% genomic DNA was desorbed from the oxidized porous silicon after five times of elution, which is the yield of DNA extracted by the microchip. That means 24 ng genomic DNA is extracted from per microlitre whole blood by using oxidized porous silicon microchip that reaches the level of commercial kits which can extract 20–30 ng DNA from per microlitre whole blood.

The extraction of genomic DNA from a crude biological sample must be PCR amplifiable. The extracted genomic DNA from whole blood by oxidized porous silicon microchip was submitted as the template for PCR to evaluate the quality of the purified DNA. The extracted DNA by a commercial kit was used for positive control and TE buffer without DNA was for negative control. All the amplified results were identified by gel electrophoresis separations. The result is shown in Fig. 4, a 203-bp fragment of -*gapd* gene is successfully amplified. This illustrates that the method for extracting DNA by microchip can effectively eliminate PCR inhibitors in whole blood and the eluted DNA can be successfully used for PCR reaction and achieve the equal efficiency of amplification of the positive control.

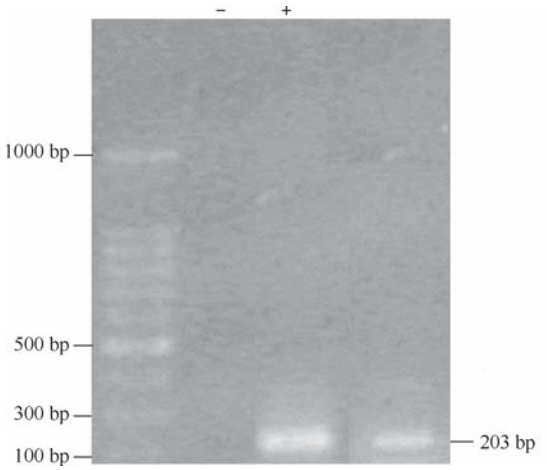


Fig. 4 Electrophoresis pattern of PCR product

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