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Fabrication and Characterization of Paper-Based Microfluidics Prepared in Nitrocellulose Membrane By Wax Printing

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Paper-based microfluidics is a promising technology to develop a simple, low-cost, portable, and disposable diagnostic platform for resource-limited settings. Here we report the fabrication of paper-based microfluidic devices in nitrocellulose membrane by wax printing for protein immobilization related applications. The fabrication process, which can be finished within 10 min, includes mainly printing and baking steps. Wax patterning will form hydrophobic regions in the membrane, which can be used to direct the flow path or separate reaction zones. The fabrication parameters like printing mode and baking time were optimized, and performances of the wax-patterned nitrocellulose membrane such as printing resolution, protein immobilization, and sample purification capabilities were also characterized in this report. We believe the wax-patterned nitrocellulose membrane will enhance the capabilities of paper microfluidic devices and bring new applications in this field.

Paper-based microfluidics (or lab on paper) is a newly developed technology which utilizes paper as the fabrication substrate to pattern microstructures on it to generate complex microfluidic functions.[1−9] It aims at developing a simple, inexpensive, portable, and easy-to-use diagnostic platform. Microfluidic devices prepared in paper have several attractive features such as low cost, ease of use, portability, and disposability. Paper patterned with microstructures can perform multiple biochemical reactions simultaneously without cross-contamination. The paper substrate ensures the whole system can be operated without an external power source. Downsizing of the reaction zones will minimize the consumption of precious chemical reagents and limited sample. So it is an ideal platform to develop low-cost, portable diagnostic devices for resource-limited regions and remote settings. It has gained more and more attention recently.[10−14]

Currently, the reported works can be summarized into two categories. (1) Fabrication: Whitesides and co-workers pioneered the field by patterning the chromatography paper through photolithography. They produced paper microfluidic devices with photoresist SU8 for simultaneous detection of glucose and protein.[1,2] Recently they extended this method to fabricate three-dimensional microfluidic devices in layered paper and tape, which are quite suitable for use in distributed healthcare and environmental screening (such as water analysis).[5] Besides the photolithography method, they also described the use of the plotting method to generate foldable paper microfluidic devices with hexanes-dissolved PDMS.[4] Abe et al. presented an inkjet printing method for the fabrication of entire paper microfluidic devices, which can also be used to print necessary reagents into predefined areas of the patterned paper microfluidic devices.[6] Li et al. demonstrated the fabrication of paper microfluidics by plasma treatment, in which they first hydrophobized paper and then treated it using plasma in conjunction with a mechanically fabricated metal mask.[7,8] (2) Detection: Whitesides and co-workers have demonstrated two optical detection methods to quantify colorimetric assays on paper microfluidics devices. One was to use a camera phone to realize on-site detection and off-site diagnostics.[3] The other was to use a hand-held optical colorimeter for quantifying the concentration of analytes by measuring the transmission of light through paper.[15] Dungchai et al. reported the first demonstration of electrochemical detection for paper-

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based microfluidic devices with the determination of glucose, lactate, and uric acid in biological samples using oxidase enzyme.[16]

Besides these works, we had previously reported a simple and low-cost fabrication method to pattern microstructures with wax on filter paper (made from pure cellulose) for bioassay, such as BSA and glucose assay.[9] The wax-patterning method we introduced included (i) painting with a wax pen, (ii) printing with an inkjet printer followed by painting with a wax pen, and (iii) printing by a wax printer directly. The wax-patterning method has several merits such as a simple fabrication process (printing and baking), fast production speed (5−10 min), low cost (both wax and paper are cheap and easy to obtain), and being environmentally friendly (no use of organic solvents throughout the fabrication process; paper and wax can be easily disposed of by burning). Thus, the wax-based micropatterning technology will be very useful for prototyping paper-based microfluidic devices to implement low-cost bioassays in remote settings.[17]

All these previous studies have laid a solid foundation to further develop the paper microfluidic technology. However, the reported paper microfluidic devices were generally fabricated in paper made from pure cellulose. It is not an ideal substrate for protein immobilization related applications, such as immunoassay and enzyme activity screening, whereas nitrocellulose membrane (NC membrane), which is a kind of paper substrate made from pure cellulose nitrate, owns high protein-binding capability due to both charge−charge interactions and weak secondary forces (van der Waals, particularly hydrogen interactions).[18] Its porous three-dimensional (3D) structure makes it an ideal protein immobilization substrate, and it is now widely used in a lot of applications, for example, Western blotting,[19] dot ELISA (enzyme-linked immunosorbent assay),[20] and gold nanoparticle based test strips.[21] Paper microfluidic devices fabricated in NC membrane have several favorable merits: (1) NC membrane is a widely used substrate for protein immobilization related applications, so those applications can be directly applied onto paper microfluidic devices fabricated in an NC membrane without further modification in substrate material. (2) The pore size of the NC membrane is quite small (0.45 μm) and uniform; thus, the wax penetration process during baking is much slower and can be controlled more precisely. So the wax-printing method can generate a microchannel in paper down to 100 μm resolution (the resulting width), which is comparable to the photolithography method (which usually consists of coating, prebaking, exposure, postbaking, developing, and hard-baking steps). (3) The pore size of the NC membrane is 0.45 μm, so micrometer-sized contaminants can be purified on the patterned NC membrane. (4) The surface of the NC membrane is much smoother, and the pore size is more uniform; thus, the flow in the NC membrane microchannels is more stable and reproducible. Here in this report, we apply the wax-printing method to fabricate paper-based microfluidics in NC membrane. Wax can form hydrophobic walls in the NC membrane, which can either form channels to direct the flow or form independent reaction zones. The production process has two main steps (printing and baking) and can be finished within 10 min. The printing mode and baking time were optimized in this work, and its uses in protein pattern, dot immunoassay, and sample purification were also demonstrated.

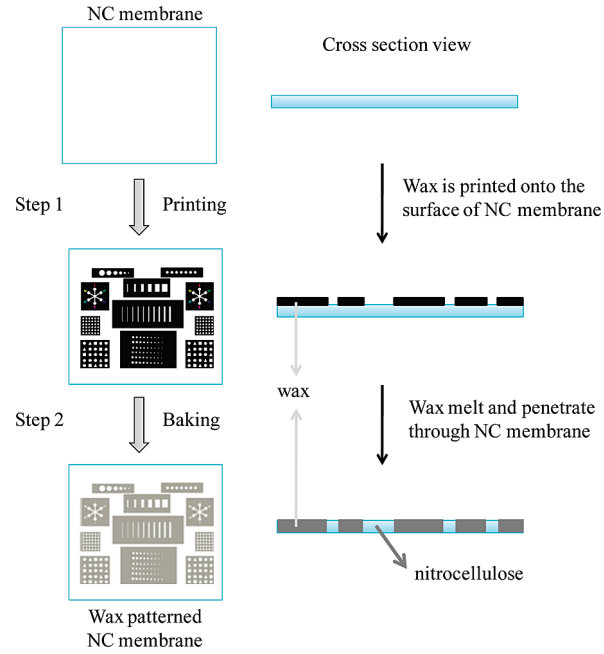

Figure 1. Schematic illustration of processes to fabricate paper-based microfluidics in an NC membrane by wax printing. It includes mainly two steps: step 1 is to print the wax pattern onto the surface of the NC membrane with a wax printer; step 2 is to bake the wax-printed NC membrane in an oven of 125 °C for 5 min to let the wax melt and penetrate through the membrane fully.

EXPERIMENTAL METHODS

Materials. Three different brands of NC membrane (0.45 μm pore size) were purchased from PALL (U.S.A.), Amersham (Amersham Biosciences, Pharmacia, U.S.A.), and Whatman (Protran, GE, U.S.A.), respectively. The type of wax printer used is a FUJIXEROX Phaser 8560DN (Japan). The baking equipment is an oven from Shanghai Permanent Science and Technology Company (PH-030A, China). Human IgG (hIgG), goat antihuman IgG (g-hIgG), and FITC-labeled goat antihuman IgG were obtained from Boruide Company (Dalian, China). Gold nanoparticle labeled g-hIgG (15 nm) was bought from Boaosen Biotech. (P. R. China). Bovine serum albumin (BSA) was purchased from Roche (Switzerland). Silver enhancer kit (SE-100) was purchased from Sigma (U.S.A.). Deionized (DI) water was supplied by Wahaha Company (Hangzhou, Zhejiang, China). All the chemicals were used as received without further purification.

Fabrication of Paper-Based Microfluidics in NC Membrane. The fabrication process is shown in Figure 1, which includes mainly two steps after designing different patterns on the computer with a mapping software: (1) print the wax microstructures onto the surface of the NC membrane with a wax printer (Supporting Information Figure S1; be cautioned that static electricity may cause problems of paper jamming sometimes, so be sure to eliminate the static electricity in the membrane before

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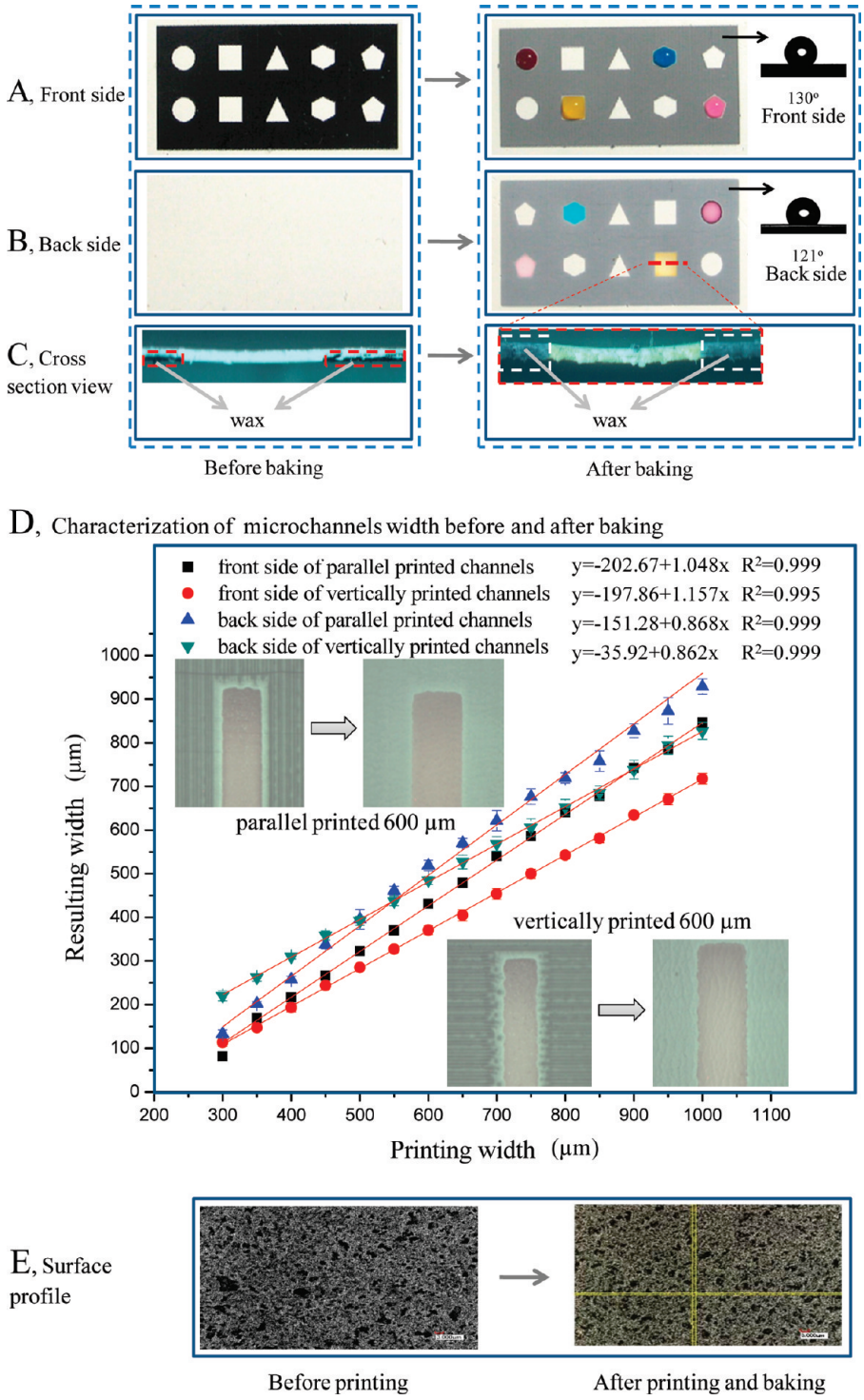

Figure 2. Comparisons of NC membranes before and after baking. Panels A and B are the comparisons of the front side image and back side image of a wax-printed NC membrane before and after baking. The contact angle measurements on the front and back side surface indicated the full penetration of wax through the membrane; panel C is the cross-sectional view of the printed NC membrane before and after baking; panel D is the characterization of the microchannels' widths (each side of the parallel-printed and vertically printed microchannels) before and after baking (the microchannels were wetted with red dye solution after baking; each point was the average value of nine points from three experiments); panel E is the comparison of the pore size of the NC membrane before and after patterning (the scale bare is 3 μm).

printing); (2) bake the wax-printed NC membrane in an oven of 125 °C for 5 min to let the printed wax melt and penetrate through the NC membrane to form the hydrophobic patterns. These two steps can be finished within 10 min and will produce the wax-patterned NC membrane. It will be ready for use after cooling to room temperature (within 1 min). We should note that the NC membrane is highly flammable (flash point is approximately 200 °C), so it should be handled with care and attention.

Immunoassay on a Wax-Patterned NC Membrane and Data Analysis. Immunoassay procedures on the wax-patterned NC membrane are similar as the sandwich ELISA format. It utilizes the 3D structure of the NC membrane as the solid support

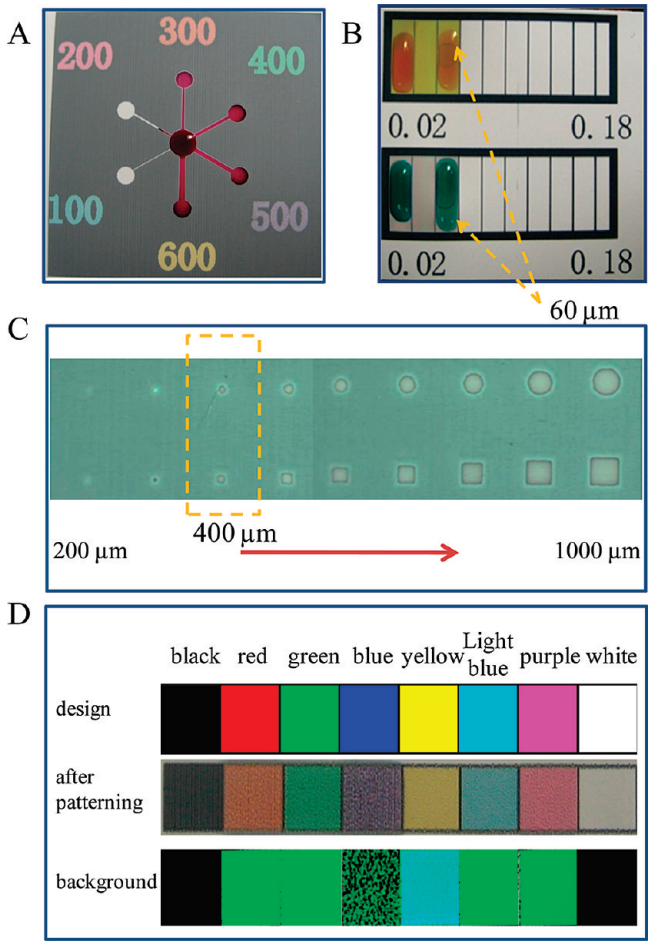

Figure 3. Characterization of a wax-patterned NC membrane. Panels A–C are the characterizations of microchannel resolution, minimal hydrophobic line width, and smallest round and square pattern obtained in the NC membrane; panel D is the characterization of background fluorescence of different colors (CCD exposure time, 1 s; 2× objective; excitation, 488 nm; collection, 525 nm).

to detect the model analyte human IgG. The immunoassay procedures included (1) coat the reaction zones with antihuman IgG; (2) block the NC membrane with 5% BSA solution; (3) human IgG with different concentrations was pipetted to react with immobilized antibody; (4) add FITC-labeled antihuman IgG or gold nanoparticle labeled antihuman IgG to form the immunocomplex on the membrane. The wax-patterned NC membrane was washed with PBST buffer (PBS + 0.05% Tween 20) three times before each new reagent was introduced. For fluorescence immunoassay, the results were imaged by an inverse fluorescence microscope (Olympus IX 71, Japan). (Exposure time of the CCD was varied according to the sample concentration.) For gold nanoparticle labeled immunoassay, the results were amplified by gold nanoparticle enhanced silver staining to transform the nanoscale probe into silver precipitation film which can be recorded by a flatbed scanner. (Before silver staining, it is necessary to wash the wax-patterned NC membrane with double-distilled water to remove unbonded gold nanoparticle labeled protein and avoid the interference of ions.) The immunoassay results captured by fluorescence microscope and desktop scanner can be analyzed using image processing and analysis software (Image-Pro, Media Cybernetics, U.S.A.) by converting each image to 8-bit grayscale and measuring the average grayscale values within the reaction zones (Supporting Information Figure S2).

RESULTS AND DISCUSSION

Fabrication of Paper Microfluidics Devices in NC Membrane. NC membrane from Whatman (BA 85, 0.45 μm pore size) was chosen as the substrate to produce the patterned membrane with the wax-printing method. The untreated NC membrane was first cut to the desired size and located into the paper box 1 in the printer. Print the wax microstructures onto the surface of NC membrane in high-resolution printing mode, and bake the wax-printed NC membrane at 125 °C in the oven for 5 min. (The detailed descriptions about the choice of NC membrane, printing mode, and baking time optimization can be found in the Supporting Information, Figure S3.) The whole production process can be finished within 10 min without use of organic solvents. Thus, the original structures of the NC membrane will be retained.

The use of the wax-printing method to fabricate paper-based microfluidic devices in an NC membrane owns several merits such as an easy production process, fast production speed, minimal material cost, and no use of organic solvents (which will minimize its interference to the structures and properties of the paper substrate). So it can be easily adapted to produce paper microfluidic devices in NC membrane at a large scale.

Characterization of the Wax-Patterned NC Membrane. Figure 2 is the characterizations and comparisons of the NC membrane before and after the fabrication process. Parts A and B of Figure 2 are the comparisons of the front side and back side images, in which we can see that the darkness of the front side decreased while the back side increased. This indicated that the wax printed onto the NC membrane melted and has penetrated through the membrane. After baking at 125 °C for 5 min, the contact angle of the back side increased to 121° ($n = 3$), which verified that the wax has penetrated into the back side. The cross-sectional view also demonstrated its full penetration (Figure 2C).

Figure 2D is the characterization of the microchannels' widths before and after baking (front side and back side), from which we can see the melted wax will not only penetrate vertically but also flow laterally during the baking process. The wax spreading process can be characterized by Washburn's equation, which describes capillary flow in porous materials:[22]

$$L = \sqrt{\frac{\gamma D t}{4\eta}} \qquad (1)$$

where t is the time for a liquid of viscosity η and surface tension γ to flow a distance L into a porous material with an average pore diameter D. As the pore size of the NC membrane is 0.45 μm, the wax lateral spreading process is relatively slow in the NC membrane compared with common filter paper. So the microchannel dimension in the NC membrane can be controlled in a more precise and more reproducible manner. Besides, we can conclude from eq 1 that the lateral spreading of wax is proportional to time when all other parameters keep constant. The resulting channel width will be the original channel width minus the wax lateral spreading distances. To validate this, we designed channels varying in widths from 300 to 1000 μm (in increments of 50 μm).

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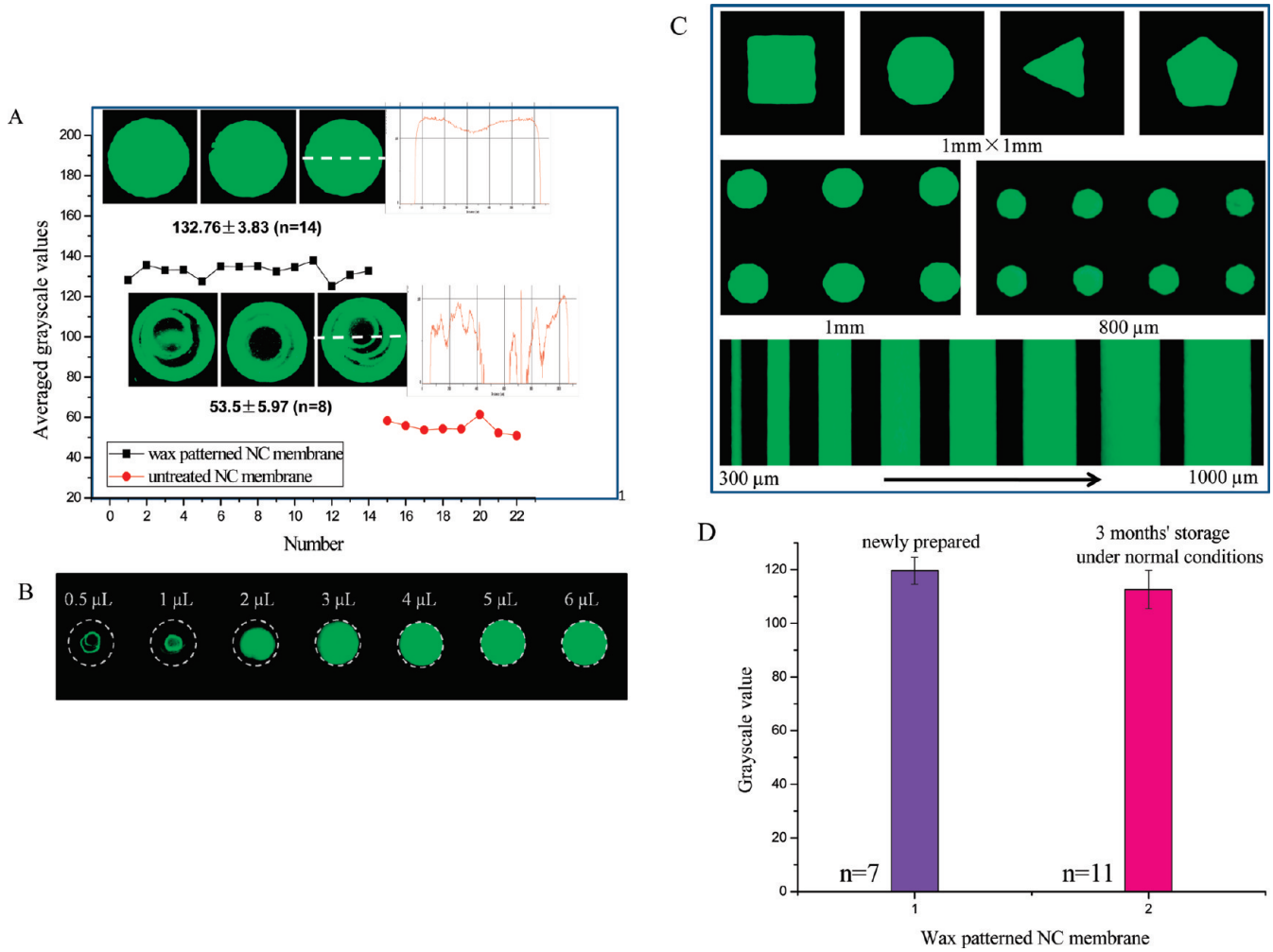

Figure 4. Wax-patterned NC membrane for protein immobilization: (A) characterization of protein immobilization on the wax-patterned NC membrane and its comparison with untreated NC membrane; (B) the liquid volume affects the uniformity of protein immobilization (3 mm diameter reaction zones); (C) different protein patterns immobilized on the wax-patterned NC membrane (the labeled dimension was the printing dimension); (D) protein immobilization on wax-patterned NC membrane stored under normal conditions for 3 months.

After being baked at 125 °C for 5 min, the lateral spreading distances of all channels were imaged with a microscope and measured with Image-Pro software (Supporting Information Figure S4). The results indicated that the spreading distance was 86 μm for parallel-printed microchannels and 118 μm for vertically printed microchannels under this condition. Thus, we can see from Figure 2D the resulting channel width (both front side and back side) was in a very nice linear relation with the printing channel width.

Figure 2E is the surface feature of the membrane imaged on a color 3D laser scanning microscope (VK-9700, Keyence, Japan), from which we can conclude that the average pore size of the NC membrane did not change during the wax printing and baking process. Its 3D structure of nitrocellulose was retained. So the wax-patterned NC membrane can be used for purifying micrometer-sized contaminants in samples.

The fabrication resolution of wax-patterned NC membrane was also characterized. Six straight microchannels with different dimensions (from 100 to 600 μm, in increments of 100 μm) were branched out of a 3 mm diameter circle. Then red dye solution (amaranth) was added into the center reservoir, and the solution would flow outward. From Figure 3A we can see the thinnest channel the red solution can pass is 300 μm (the resulting channel width is about 100 μm), which can be deemed as the highest resolution the wax-printing method can achieve in the NC membrane. We also characterized the smallest wax line width to confine the liquid by separating the grids with the wax line varying in width from 20 to 180 μm (in increments of 20 μm). From Figure 3B we can see even the 60 μm wax line can still function well as the hydrophobic barrier after baking. The smallest round pattern and square pattern generated with the wax-printing method is 400 μm. Background fluorescence of different colors was also tested in our experiments (black, red, green, blue, yellow, light blue, purple, and white). The results told us that black color has barely background fluorescence, so it is the color of choice when background fluorescence should be avoided. The colors owning background fluorescence may be used as an internal fluorescence control or a fluorescence identification label.

After the wax-patterned NC membrane was fabricated, it can be stored under normal conditions for further use. We also kept the solid wax ink (Xerox, Japan) on a filter paper (102 or 202, Hangzhou Xinhua Paper Limited, China) in an oven of 80 °C for 40 min and did not observe any penetration in the filter paper. This indicated that the wax-patterned NC membrane can be stored under 80 °C, which is sufficient for common use.

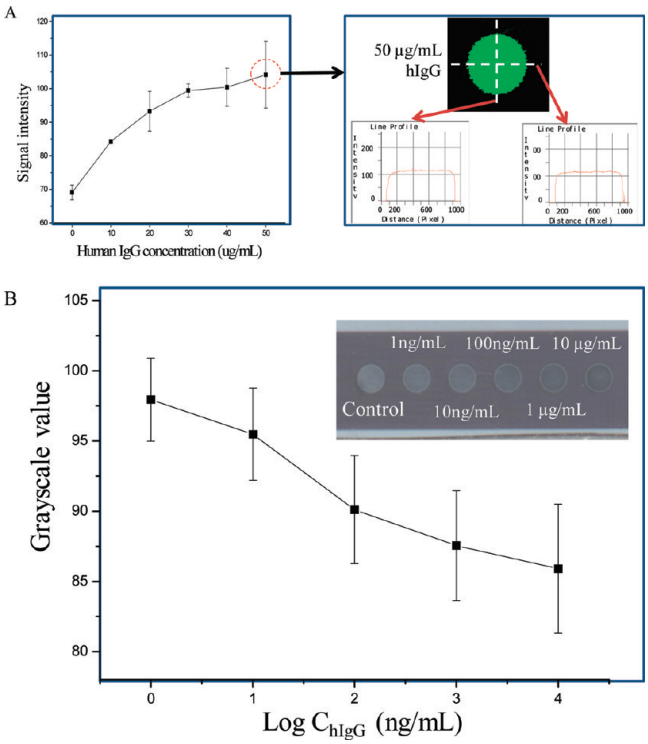

Figure 5. Wax-patterned NC membrane for immunoassay: (A) the calibration curve for human IgG (sandwich immunoassay format, $n = 3$) and the signal uniformity analysis within the immunoassay zone of 50 μg/mL human IgG; (B) colorimetric immunoassay with silver staining enhanced gold nanoparticle labeled immunoassay for human IgG.

Wax-Patterned NC Membrane for Protein Pattern. After the NC membrane was patterned (printing and baking) with wax designs, its protein immobilization characterization was conducted. A 2 μL 40-fold dilution of FITC-labeled goat-antihuman IgG was pipetted onto a 2 mm diameter reaction zone confined by the patterned wax and then kept in a humid box for 3 h under room temperature. The wax-patterned NC membrane was put onto a PDMS slab to avoid the cross-contamination through the bottom substrate during the immobilization process. Then wash the membrane three times with PBST (PBS + 0.05% Tween 20) and image the results with the fluorescence microscope. For comparison, the same experiments were also conducted on the untreated NC membrane (the resulting diameter is about 3.38 mm). The results (Figure 4A) showed that NC membrane patterned with wax can immobilize protein as well as untreated NC membrane. Besides, the protein immobilization is very uniform on the wax-patterned NC membrane. This advantage is contributed by the confinement of wax microstructures. After the NC membrane was patterned with wax, liquid solution can be held at defined locations without cross-talk. In addition, a larger volume of liquid sample can also be loaded into specific reaction zones which can minimize the ring effect encountered in the protein pattern on the untreated NC membrane and ensure uniformity.[23] We demonstrated this by adding different volumes of FITC-labeled antibody (0.5, 1, 2, 3, 4, 5, 6 μL) into 3 mm reaction zones to coat the NC membrane for 3 h. (The protein immobilization will reach equilibrium within 3 h, Supporting Information Figure S5.) The results (Figure 4B) showed that when the pipetting volume exceeds 4 μL, the uniformity of the reaction zone can be ensured. Thus, the wax-patterning method here can be used for fabrication of low density of a protein array with uniform intensity. Besides, different sizes and designs (shown in Figure 4C) can also be made with our method. In comparison with the traditional plotting and pipetting method, which can only generate round patterns, this method can generate different patterns, such as squares, triangles, pentagons, hexagons, etc. So the wax patterning in the NC membrane can also be treated as a flexible plotting method.

We also characterized the protein immobilization performance on the wax-patterned NC membrane stored for 3 months under normal conditions at room temperature. It did not show any decrease in protein immobilization capability compared with freshly prepared wax-patterned NC membrane (Figure 4D).

Wax-Patterned NC Membrane for Dot Immunoassay. We then demonstrated its use in dot immunoassay with human IgG as a model analyte. Both fluorescence immunoassay and colorimetric immunoassay (gold nanoparticle immunoassay coupled with silver staining which can be monitored by naked eyes or quantified by desktop scanner) were demonstrated. The reaction zones were 3 mm diameter round patterns, and the results are shown in Figure 5 (the optimizations of immunoassay conditions are shown in Supporting Information Figure S6). The wax-patterned NC membrane for dot immunoassay has several merits: (1) Conventional dot immunoassay uses a pencil to draw grids on the NC membrane to direct the addition of reagents and samples to the precise locations. However, this method cannot ensure adding reagents and samples sequentially to a specific location in a reproducible manner and the nearby samples may cross-talk with each other sometimes, whereas for the wax-patterned NC membrane these problems can be eliminated as the wax microstructures will stop the spreading of liquids. (2) The ring effect encountered in the dot immunoassay can also be minimized due to the confinement of the hydrophobic pattern. The immunoassay signals will be more uniform (Figure 5A). (3) The antibody-immobilized patterned NC membrane can be cut into individual strips of the same reaction size. Then it may be treated as a polymer bead and processed further within tubes, 96-well plates, or other reaction vessels.

Wax-Patterned NC Membrane for Sample Purification. Purifying the contaminants from a sample is very important as they may interfere with the results. As the average pore size of NC membrane used in our experiments is 0.45 μm (1 or 2 orders of magnitude smaller than common filter paper), its purifying ability was enhanced. We demonstrated this by adding a 10 μL solution of 1 μm silica particles (MagPrep, Merck, Germany) (50, 5, and 0.5 mg/mL, respectively) and red dye solution (amaranth, MW = 604.480) into paper microfluidic devices. From Figure 6 we can see that red dye molecules and 1 μm particles can both pass through patterned filter paper, whereas for comparison, 1 μm particles cannot pass through the wax-patterned NC membrane. The results suggested that the wax-patterned NC membrane can be used to purify micrometer-sized impurities, such as beads and cells. So we can add sample from one side of the NC membrane and perform reactions on the other side to eliminate the interferences of impurities. The filtrated solution can also be collected by another piece of hydrophilic paper for further

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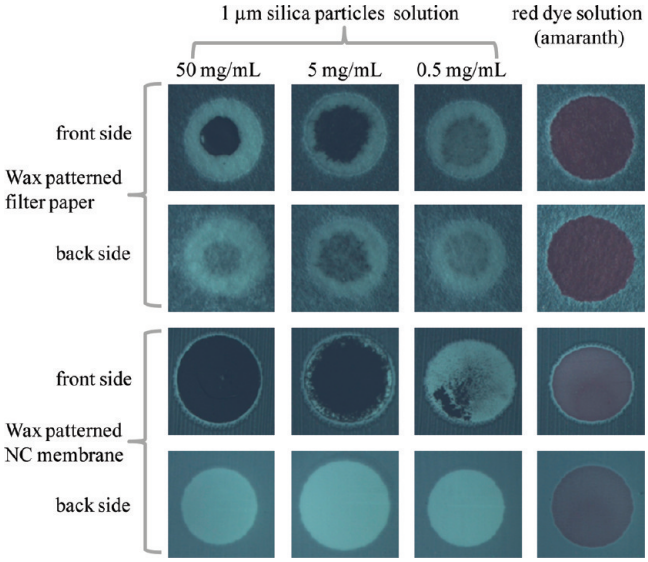

Figure 6. Characterization of the sample purification performance of a wax-patterned NC membrane using 1 μm silica particles and red dye solution (amaranth) and its comparison with wax-patterned common filter paper.

experiments (Supporting Information Figure S7). But we should state here that, although proteins can pass through the wax-patterned NC membrane, the concentration was not the same on each side. Only part of protein molecules can pass through the pores in the NC membrane without external force.

CONCLUSIONS

In conclusion, we introduced the fabrication of paper microfluidic devices in NC membrane by a wax-printing method. The whole production process includes mainly printing and baking steps and can be finished within 10 min without the use of organic solvents. Thus, the wax-patterned NC membrane can be produced at a large scale with such a method. Due to the high protein-binding capability of the NC membrane, paper microfluidic devices in the NC membrane will facilitate protein immobilization related applications like bioactive paper sensor,[11−14] dot ELISA,[20] protein array,[24] and paper microzone plates.[25] In addition, the wax-patterned NC membrane also offers more flexibility and capability in sample manipulation over untreated NC membrane owing to the confinement of wax microstructures. We believe it will open new possibilities and bring broad applications in clinical diagnosis, food safety inspection, and environmental screening.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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