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Multiplexed Proteomic Sample Preconcentration Device Using Surface-Patterned Ion-Selective Membrane

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Abstract

In this paper, we report a new method of fabricating a high-throughput protein preconcentrator in poly(dimethylsiloxane) (PDMS) microfluidic chip format. We print a submicron thick ion-selective membrane on the glass substrate by using standard patterning techniques. By simply plasma-bonding a PDMS microfluidic device on top of the printed glass substrate, we can integrate the ion-selective membrane into the device and rapidly prototype a PDMS preconcentrator without complicated microfabrication and cumbersome integration processes. The PDMS preconcentrator shows a concentration factor as high as $\sim 10^4$ in 5 min. This printing method even allows fabricating a parallel array of preconcentrators to increase the concentrated sample volume, which can facilitate an integration of our microfluidic preconcentrator chip as signal enhancing tool to various detectors such as mass spectrometer.

INTRODUCTION

In proteomics, the large dynamic range of the protein concentrations (from few pg/mL up to mg/mL), as well as the large number of the proteins ($> 100,000$) in a given sample pose a huge challenge to sensing.¹ To reduce the sample complexity, the high-abundance proteins have to be sorted. At the same time, the low-abundance molecules have to be concentrated, since the concentration of low-abundance ones might fall well below the detection limit of biosensors, especially after several separation steps. The preconcentration step is even more important when using microfluidic sensing systems because of the inherently small channel sizes, which usually lead to inefficient use of available target molecules in the given samples.²

There have already been several preconcentration schemes developed both in capillary electrophoresis^{3–7} and in microfluidic chip format.^{8–16} Among these various preconcentration schemes, electrokinetic trapping offers one significant advantage due to its general applicability: any charged molecules or particles, regardless of their sizes, can be effectively captured and concentrated in diverse buffer conditions. Our group has been working on a novel micro/nanofluidic preconcentration chip based on electrokinetic trapping. It consists of two microchannels bridged by a number of nanochannels acting as robust ion-selective membrane.¹³ Even though it showed impressively high preconcentration efficiency, it required a fabrication of the nanochannels and the concentrated sample volume was limited to few picoliters due to the small channel size, typically only a few μm in channel depth. This small sample volume would not be an issue if the concentrated plug is only processed inside the microchannel for further operations such as on-chip immunoassay.^{17, 18} If, however, the concentrated sample plug has to be sent to external sensing units such as mass spectrometer,

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its volume needs to be in the nanoliter range.^{19–21} To address the issues of fabrication and limited sample volume, techniques have been developed to implement electrokinetic concentration scheme in a poly(dimethylsiloxane) (PDMS) microfluidic chip format.^{9, 16} However, the non-reproducible bonding strength between glass substrate and PDMS chip, as well as the limited permselectivity of the nanojunctions, were limiting the yield and efficiency of the preconcentrator device. More recently, Lindberg et al. have developed a planar microfluidic preconcentrator by integrating a thin Nafion membrane sheet, a highly ion-selective membrane normally used in proton-exchange membrane fuel cells.²² Using the “electrocapture” method, they could demonstrate a preconcentration of microbeads. However, an integration of such a thin film material (127 μm thick) into microfluidic systems is generally challenging, and it is not easily applicable to building a parallel array of preconcentrators. To combine the distinctive advantages of the PDMS material such as the easiness of fabrication and simple bonding procedure with the high ion selectivity of the Nafion material^{23, 24}, we came up with a simple fabrication strategy. Instead of integrating a thin Nafion film between two halves of the device, we print a submicron-thick ion-selective material on a glass substrate and plasma bond the PDMS microfluidic chip on top of the surface-patterned glass substrate. The basic concept of this method is shown in Figure 1. Through the standard plasma bonding process, the planar ion-selective membrane printed on the glass substrate can be easily integrated into the device. It is simply embedded below the deformable PDMS chip by plasma bonding. However, there is a limitation of this approach in terms of the thickness of the printed membrane up to which the PDMS can be deformed and seal the membrane. Furthermore, this fabrication method allows arranging many preconcentrators in an array. This arrayability enables to increase the volume of the concentrated sample sufficiently enough to directly interface the preconcentrator to an external sensing unit such as mass spectrometer.

EXPERIMENTAL SECTION

Design and Fabrication

The layout and operation schemes of a PDMS protein concentration chip with surface-patterned ion-selective membrane, such as Nafion[®] membrane, are shown in Figure 2. Nafion is well-developed ionomer by the DuPont company, showing a high ionic permselectivity due to cluster network²⁵ as well as due to sulfonate groups.^{26, 27} Nafion junction in this device functions as the nanofluidic channels of our previous silicon nanochannel device, but with potentially higher permselectivity due to smaller pore size and charge density. The chip consists of 2 microchannels (See Figure 2a); the middle channel is filled with a proteomic sample that has to be concentrated and the outer channels, which surrounds the sample channel left and right in the middle section of the device, are filled with a standard buffer solution. Instead of having just one buffer channel running parallel to the sample channel, it is more advantageous to have such a dual gate structure with respect to the stability of the depletion region.²⁸ In this dual gate design, the depletion region can be formed from both sides of the sample channel, increasing the stability of the depletion region. The embedded planar Nafion membrane connects three microchannels at the bottom of the channels as a permselective membrane (Figure 2b). The operation of the device is performed in depletion and concentration mode, as shown in Figure 2c. First, we generate a depletion region by applying a potential difference (e.g., 50 V) between the sample channel and the buffer channel through the planar ion-selective membrane. Once the depletion region has been formed and stabilized, a potential difference is applied across the sample channel to inject the molecules by electroosmotic flow to the depletion zone and electrokinetically trap them in front of the depletion region.^{13, 16} The detailed fabrication steps of the device, including the surface patterning of the permselective membrane on a glass slide, are shown in Figure 3. For patterning, Nafion[®] perfluorinated ion-exchange resin (Sigma-Aldrich, St. Louis, MO) was used. It is a 5 wt % solution in lower aliphatic alcohols/H₂O mix and contains 15–20% water. Due to this high content of water, it

has a low viscosity and therefore, easy to flow inside the microchannel. In principle, any ion-selective resins with reasonably low viscosity can be used for surface patterning. As for the patterning method, there are two alternative methods available for non-photolithographic methods, as shown in Figure 3a and Figure 3b. In the first method, a PDMS microstamp is used to print the membrane.²⁹ The major steps are: (1) ink the PDMS microstamp with Nafion resin, (2) transfer Nafion resin to a glass substrate, and (3) cure the patterned Nafion resin on a hotplate at 95 °C for 10 min. To achieve a good wetting of the Nafion resin on the PDMS stamp, we treated the surface of the PDMS stamp with plasma beforehand. For a good printing result, the cleanness of the glass substrate is important. It has to be free of any particles and dusts. We used standard pre-cleaned microscope glass slides (25mm × 75mm) and cleaned the surface with IPA and DI water thoroughly before printing. It turned out that the microcontact printing with a consistent thickness of the Nafion membrane between 200–400 nm was quite challenging. Depending on the pressure applied on the stamp and the pressure distribution when stamping on the glass surface, the thickness of the Nafion film varied between 0.5–1 μm. Nafion films that are too thick might develop leakages in the final, bonded device, therefore should be avoided. As an alternative patterning method, we applied the microflow patterning technique which utilizes the PDMS microchannel (reversibly bonded to glass) to define the flow path of the resin (Figure 3b). The channel geometry used was 120 μm deep and 200 μm wide. By placing a drop of liquid Nafion resin at one open reservoir of the channel, the channel was immediately filled with it by the capillary force (step 1 in figure 3a).³⁰ We typically used 1 μL of the resin. Then, the liquid resin was completely flushed out of the microchannel by negative pressure applied through the syringe tubing on the other end of the channel (step 2 in Figure 3b). After this step, only a thin layer of Nafion remained on top of the hydrophilic glass slide. In this flow patterning process, the thickness of the membrane is mostly controlled by the amount of the applied negative pressure. A repeatable thickness can be achieved as long as a constant pressure is applied through the tubing. To fully cure the patterned ion-selective film in a stripe shape (3 in Figure 3b), it was heated up at 95°C for 10 minutes. Finally, the PDMS chip with microchannels was bonded on top of the patterned glass substrate by standard plasma bonding (4 in Figure 3b). Since an exact alignment was not crucial, the bonding could be performed without any help of microscope. To enhance the bonding strength, the assembled device was heated up on a hotplate at 70 °C for 12 hours.

Materials and Measurement Set-Up

We used a β-Phycoerythrin (β-PE protein; MW 240kDa and pI 4.3) from Sigma-Aldrich Chemical Co (St. Louis, MO) for the characterization of the PDMS device. To investigate the influence of the voltage difference across the microchannel and the ion-selective membrane, we used 10 nM β-Phycoerythrin in a pH 7 phosphate buffer. The fluorescence signal was measured after 1 min. of preconcentration. To measure the preconcentration speed, we prepared four different concentrations of β-Phycoerythrin, 40 pM, 400 pM, 4 nM, and 40 nM, using a 10⁻² M phosphate buffer at pH 7. We also measured the fluorescence intensities of three samples with known concentrations (2, 4, 8 μM) and used them as internal standards. Measurement with different initial concentrations of β-PE protein provided a direct comparison of the preconcentration factor with our previous result.¹⁶

An inverted epifluorescence microscope IX 71 (Olympus, Melville, NY) equipped with a thermoelectrically cooled CCD camera (Hamamatsu Co., Japan) was used for fluorescence imaging. Fluorescence images were taken every 30 sec and were analyzed using IPLab software (Scanalytics, Fairfax, VA). To reduce the photobleaching effect, we used a mechanical shutter (Olympus, Melville, NY) which automatically blocks UV light and opens only when images are taken. Also, we used a neutral density filter (Olympus, Melville, NY) to prevent the CCD array from saturation so that we can achieve an increased dynamic range and reduce the photobleaching effect.

RESULTS AND DISCUSSION

The PDMS preconcentrator chip is shown in Figure 4a. The sample channel is marked as red and the buffer channel as green. All the microchannels were 50 μm deep and 50 μm wide. The overall size of the device was 20 mm by 25 mm. In Figure 4b, the scanning electron microscope (SEM) images of a Nafion membrane on a glass substrate are shown which was patterned by the microflow patterning technique. A well-defined stripe was formed on the glass substrate and the thickness of the Nafion membrane on glass was 191 nm. As shown in the cross sectional view of the assembled device in Figure 4c, the elastic PDMS chip conformed adequately to the rounded shape of the patterned ion-selective membrane at this thickness. With increasing thickness of the membrane above 400nm, however, the membrane wouldn't be sealed by the PDMS device, leaving leakage holes at the edges of the membrane. This imperfect sealing would compromise the ion selectivity of the membrane and consequently, the preconcentration efficiency and reproducibility of the device.

The single most important parameter in the preconcentration experiment is the voltage difference across the sample channel ($V_{\text{diff}} = V_1 - V_2$) which also determines the voltage difference across the ion-selective membrane. We measured the preconcentration factor (Y_1 ; left Y axis) and the size of depletion region (Y_2 ; right Y axis) of β -PE protein as a function of the voltage difference V_{diff} , as shown in Figure 5. For each measurement, V_1 was set to $V_1 = 2V_2$. The preconcentration factor as well as the size of the depletion region increased with higher voltage difference. The four insets taken at four different voltage difference show that the size of the depletion region measured as the distance of the concentrated plug from the location of the ion-selective membrane increased with higher voltage difference. The intensity of the concentrated plug also increased. This result can be explained with the fact that the depletion force as a function of the normal field (E_n) across the membrane was increased with the voltage difference V_{diff} and moved the charged molecules farther away from the membrane towards the reservoir with a higher potential V_1 . At the same time, the voltage difference V_{diff} increased the tangential flow as a function of the tangential field (E_t) which generates the electroosmotic flow acting against the increasing depletion zone. It moves the molecules towards the depletion zone and stacks them in front of the depletion zone. Eventually, these two field components, E_n and E_t , find a balance which leads to a formation of a stable concentrated plug in a certain distance Y_2 to the Nafion membrane. Theoretically, we could continuously increase the voltage difference V_{diff} to acquire higher preconcentration factor. The stability of the concentrated zone, however, was compromised by further increasing the voltage difference over 25V, since the zone was affected by the secondary nonlinear electrokinetics near the nanochannels.²⁸ For this reason, we set the optimum voltage difference V_{diff} to 25 V ($V_1 = 50\text{V}$, $V_2 = 25\text{V}$).

To quantify the preconcentration factor and speed at this voltage difference V_{diff} , we measured the fluorescence intensity of collected B-phycoerythrin (β -PE) protein as a function of time for four different starting concentrations (40 pM, 400 pM, 4 nM, and 40 nM). The experimental data shown in Figure 6 were taken from the average values out of three devices. The coefficient of variance (C.V.) was 11.5%, showing a reasonable reproducibility of our devices given the inaccuracy of pattern alignments in the fabrication process. At an initial concentration of 40 nM, the intensity reached the saturation value only after 2 min. The inset fluorescent images in Figure 6 were taken at three different preconcentration durations in case of 4 nM, validating the increasing intensity with preconcentration time. In case of 400 pM, the concentration factor reached the reference intensity of 8 μM ($\sim 2 \times 10^4$ fold increase) just within 5 min. (see the animation in the Supporting Information 1). At the lowest initial concentration tested, 40 pM, a similar order of concentration increase ($\sim 5 \times 10^4$ fold increase) was reached within 10 min. and enabled a fluorescence detection despite of such a low protein concentration. The concentration speed was seemingly not constant, and furthermore, it seemed to be a function

of concentration of the plug. While the exact cause for this is uncertain, we hypothesized that non-specific binding and loss of molecules to the wall, which is supposed to be more significant for higher-concentration plugs, might be the reason.

This concentration speed was greatly higher than that from our PDMS preconcentrator fabricated by the junction gap breakdown method¹⁶, which showed a preconcentration factor $\sim 10^4$ within 1 h. Another important result from this measurement was the increased concentration volume of β -PE as high as 200 pL. This was 350 folds larger than that achieved with silicon nanofluidic preconcentrator.¹³ The fact that we used the PDMS device also contributed to this increase. Since there is no voltage limit imposed by the breakdown of the oxide layer, significantly higher voltage could be applied to increase the depletion force and tangential flow. This comparison with previous devices demonstrates that our PDMS preconcentrator with an embedded planar ion-selective membrane is highly efficient in terms of speed and concentrated sample volume. This increase in speed and volume was enabled by the high ionic permselectivity of Nafion which in turn is due to a cluster network²⁵ reducing the effective channel size as well as due to the sulfonate groups^{26, 27} increasing the negative charge density.

Since the surface patterning technique enables a great flexibility in depositing membranes anywhere on the glass substrate, a parallel array of preconcentrators can be easily built. The arrayability of the preconcentrator is highly important with respect to the interfacing of the preconcentrator with external sensing equipment, such as mass spectrometry. Such equipments usually require a concentrated sample volume of around few hundreds of nL. To demonstrate the arrayability of our planar PDMS preconcentrator, we built an array containing 10 single preconcentrators, as shown in Figure 7. The single preconcentrators, marked as red, were built in radial direction, so as to collect the concentrated plug of each device in the center point. The samples were loaded through each single sample reservoir, numbered from S1 to S10 and these were kept at $V_1 = 50V$. All the preconcentrators shared a single common reservoir, the sample collecting reservoir, which was kept at $V_2 = 25 V$ in order to initiate the electroosmotic flow of the sample toward the center point. A single buffer channel, marked as green, was running parallel to each sample channel. For an equal electric field condition, each preconcentrator had an individual ground reservoir fabricated along the buffer channel. The Nafion membrane was patterned in a half-circle shape on a glass substrate. The magnified view showed the electric configuration of one single preconcentrator where the Nafion membrane connected the sample channel to the buffer channels. Starting with 4 nM β -PE (see Figure 6), we successfully operated the array chip (see the animation in the Supporting Information 2). With this array, we were able to increase the preconcentrated sample volume according to the number of the single preconcentrators. In this specific case, it was 2 nL which was 3500 folds larger than that from our previous report.¹³ The same approach could be applied to increase the sample volume even more and generate sufficiently high sample volume to transfer the sample using pipette tip or send directly to a mass spectrometer (MS) via electrospray or droplet dispensing.

CONCLUSIONS

We developed high-throughput proteomic sample preconcentrator in PDMS by using a surface-patterned ion-selective membrane. Instead of integrating a Nafion film, which complicates the assembly and operation process, we simply integrated a thin printed Nafion membrane by plasma bonding between the PDMS chip and the glass substrate. The membrane was adequately sealed between the PDMS top and the glass substrate and no leakage was observed. The PDMS preconcentrator showed a high preconcentration efficiency with a factor as high as $\sim 10^4$ in just 5 min., which was $\times 12$ higher than our previous PDMS preconcentrator fabricated by junction gap breakdown. Moreover, we have demonstrated a fabrication of 10 single preconcentrators in an array format which increased the preconcentrated volume by 3 orders

of magnitude compared to our previous result obtained with the silicon nanofluidic preconcentrator. The ability of building a massively parallel array using this technique is significant in terms of the integration of our preconcentrator to an external sensing unit such as mass spectrometer. In addition to a shorter preconcentration time, the array can offer a sufficient amount of the concentrated sample volume to transfer it to an external sensing unit. Due to this capability, we expect a high potential of our PDMS preconcentrator chip as signal enhancement tool for mass spectrometer to detect low-abundance proteins and peptides. Furthermore, the PDMS microfluidic format of this device would allow the integration of preconcentrator to many different BioMEMS platforms, including cellular BioMEMS devices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Concept of the single-step integration of ion-selective membrane into PDMS preconcentrator by plasma bonding.

Figure 2.

Schematics of PDMS preconcentrator with surface-patterned Nafion membrane on the glass substrate and its operation. The middle channel is loaded with a sample and the side channel is filled with a buffer solution. For preconcentration, a potential difference applied across the middle and the side channel in combination with an electrokinetic flow.

Figure 3. Fabrication of PDMS preconcentration device via surface-patterned ion-selective membrane using (a) micro-stamping technique and (b) micro-flow patterning in microchannel.

Figure 4.

(a) Optical images of the PDMS preconcentrator chip. All the microchannels were 50 μm deep and 50 μm wide. Scanning electron microscope images of a Nafion patterned glass (b) before and (c) after PDMS bonding. The thickness of the Nafion membrane on glass was 191 nm.

Figure 5. Preconcentration factor and size of the depletion region as a function of voltage difference.

Figure 6.

Preconcentration of β -Phycoerythrin protein versus electrokinetic trapping time. This result shows that we can achieve a preconcentration factor of $\sim 10^4$ in 5 min. Fluorescence images of 4 nM protein shown next to the graph indicate an increase of the concentrated plug in size and concentration with trapping time.

Figure 7.

(a) Array of 10 single preconcentrators, (b) magnified view of a single preconcentrator with electrical configuration and (c) fluorescence images of single preconcentrators in the array.