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1   Abstract

2   We report on a fabrication and packaging process for a microsystem consisting of a mass-based

3   protein detector and a fully integrated preconcentrator. Preconcentration of protein is achieved

4   by means of a nanofluidic concentrator, which takes advantage of fast non-linear electro-osmotic

5   flow near a nanochannel-microchannel junction to concentrate charged molecules inside a

6   volume of fluid on the order of one picoliter. Detection of preconcentrated protein samples is

7   accomplished by passing them through a suspended microchannel resonator, which is a hollow

8   resonant cantilever serially connected to the nanofluidic concentrator on the same device. The

9   transit of a preconcentrated sample produces a transient shift in the cantilever's resonance

10   frequency proportional to the density of the sample, and hence the concentration of protein

11   contained in it. A device containing both nanofluidic concentrator and suspended microchannel

12   resonator structures was produced using a novel fabrication process which simultaneously

13   satisfies the separate packaging requirements of the two structures. Initial testing of this

14   prototype device has demonstrated that the integrated suspended microchannel resonator can

15   accurately measure the concentration of a bovine serum albumin sample which was

16   preconcentrated using the integrated nanofluidic concentrator. Future improvements in the

17   fabrication process will allow site-specific surface modification of the device and compatibility

18   with separation methods, which will create opportunities for its application to immunoassays and

19   universal detection.

20

1   1. Introduction

2

3   Recently, the Han research group developed a microdevice which takes advantage of fast non-

4   linear electro-osmotic flow (EOF) in the vicinity of a nanochannel-microchannel junction to

5   preconcentrate charged molecules by up to 10^4-10^6-fold in less than an hour. [1, 2] This

6   nanofluidic concentrator (NC) possesses several advantages over other biomolecular

7   preconcentration techniques.   Methods such as field-amplified sample stacking [3-6],

8   isotachophoresis [7] and micellar electrokinetic sweeping [8-10] all have buffering requirements

9   which can interfere with downstream sample processing, making them difficult to integrate with

10   detectors.   Electrokinetic trapping techniques [11-13] show promise as an efficient way to

11   concentrate proteins, but the linearity and stability of these methods is a concern.

12   Chromatographic preconcentration techniques [14-17] capture proteins by the hydrophobic

13   interaction, and hence are biased toward large hydrophobic proteins and less sensitive to smaller

14   or more hydrophilic molecules. Membrane filter preconcentration [18, 19] is another example of

15   a method which is biased toward larger molecules. Compared to these alternatives, the NC

16   method of biomolecular preconcentration is the most favorable in terms of general applicability

17   to a wide range of molecular species, ease of integration, and rate of preconcentration. Existing

18   NC devices with integrated sensing capabilities rely on fluorescent labeling of the target analyte

19   molecules for detection. This approach is not ideal, because conjugation of target proteins to dye

20   molecules can modify their chemical properties, which can interfere with sensing methods that

21   require selective capture of the target by probe molecules. In addition, because fluorescent

1   detection is subject to photobleaching, autofluorescence of the sensing environment and

2   fluctuations in the excitation source intensity, quantitative sensing often depends on the

3   repeatability of calibration protocols.

4

5   These limitations can be overcome by integrating the NC with a suspended microchannel

6   resonator (SMR) sensor, as shown in Figure 1. The SMR is a resonant cantilever with a fluid-

7   filled microchannel running through it. The resonance frequency of the cantilever changes in

8   proportion to the buoyant mass of molecules and ions added to the suspended microchannel,

9   which makes it a general platform for quantitative, label-free detection in fluid. For example,

10 universal detection has been demonstrated by prefractionating mixtures by HPLC and measuring

11 the bulk density of the column's output on an SMR. [20]   The SMR has also been used to

12 conduct label-free immunoassays in blood serum by measuring the quantity of target protein

13 captured by antibody probes attached to the surfaces of the suspended microchannel. [21].

14 The sensitivity in both bulk density and surface based detection with the SMR would be

15 substantially enhanced by integrated preconcentration using a NC for sufficiently soluble protein

16 analytes. In addition, the SMR's ability to measure the spatial and temporal dependence of ionic

17 concentrations in the vicinity of the NC may provide new information about the underlying

18 transport processes which could inform the design of future NC devices. Here we report a novel

19 fabrication process which was used to produce a microsystem containing both NC and SMR

20 structures. A monolithic system is required in order to allow the minute sample volume from the

21 preconcentrator to be transferred to the SMR without significant dispersion. Functionality of the

1   integrated system has been demonstrated by preconcentrating a model aqueous protein solution

2   using the NC, transferring the concentrated sample to the SMR, and measuring the final

3   concentration obtained by SMR densitometry. By establishing successful operation of the NC

4   and SMR simultaneously on the same device and quantitative transfer of preconcentrated protein

5   samples between them, an important first step has been taken in the development of highly-

6   sensitive universal detection systems and immunoassays.

7

8   2. Design

9

10   Integration of the NC and SMR was aided by the fact that the internal volume of existing

11   standalone SMR devices (~25 pL), is comparable to the typical volume of concentrated protein

12   samples which are produced in existing standalone NC devices (~1 pL) so that sample transfer

13   and detection can be accomplished without significant dilution of the concentrated sample.

14   Hence the physical dimensions of the NC and SMR components of the integrated device were

15   made as similar as possible to those of standalone devices which have been previously

16   demonstrated. [22, 1] The integrated NC structure consists of two microchannels with cross-

17   sectional dimensions of 15 μm x 3 μm that are connected by a series of ~40nm-deep

18   nanochannels (Figure 2). When these nanochannels are filled with media having an ionic

19   strength of ~10 mM, the Debye length is comparable with the nanochannel depth, which gives

20   the nanochannel perm-selective ion transport properties when a longitudinal electric field, E_n, is

21   applied. These nanochannels have silicon dioxide surfaces bearing negative fixed surface

1   charges at near-neutral pH, and hence positive counter-ions are transported more readily than

2   negative co-ions. This results in depletion of counter-ions from the anodic end of the

3   nanochannel and enrichment at the cathodic end. To maintain charge neutrality, co-ion

4   concentrations mirror these changes in counter-ion concentration, producing a depletion region

5   of reduced ionic strength at the anodic end of the nanochannel, a condition which is referred to as

6   ion concentration polarization. [23] If E_n is increased further, a situation may arise where the

7   diffusion of counter-ions from the bulk will not be sufficient to maintain charge neutrality in the

8   depletion region, in which case an extended space-charge layer (SCL) will form at the anodic

9   end of the nanochannel. This layer can be thought of as an extension of the Debye layer of the

10   nanochannel itself, with mobile counter-ions in the SCL screening fixed charges present on the

11   nanochannel walls. The concentration of counter-ions in the SCL, which is linearly dependent

12   on the magnitude of E_n, gives rise to non-linear electrokinetic phenomena when a tangential

13   electric field, E_t, is applied longitudinally to the microchannel at the anodic end of the

14   nanochannel. One of these phenomena is electro-osmotic flow (EOF) of the second kind [24,

15   25], which is proportional to the product of E_n and E_t, and is much stronger than EOF observed

16   ordinarily in microchannels, which scales simply as E_t. [26-28] Macromolecules are rapidly

17   transported through the anodic microchannel by this non-linear EOF, and those possessing fixed

18   charges with their own associated Debye layers are repelled from the depletion region, because

19   the low ionic strength of this region makes it energetically unfavorable. This balance between

20   strong EOF of the second kind and repulsion from the depletion region results in

21   preconcentration of macromolecules at the outer boundary of the depletion region.

22

1　　The main difference between the design of the integrated NC (Figure 2) and that of previous

2　　standalone NC devices [1] is the physical structure of the nanochannel filter. Although

3　　nanochannels in both cases were etched to the same depth of ~40nm, as determined by contact

4　　profilometry, differences in anodic bonding conditions between the two processes resulted in

5　　filters with higher ionic conductivities in the case of the integrated device. As a result, filters in

6　　the integrated device can support significant EOF with the application of a purely normal electric

7　　field. This makes it possible to achieve self-stabilizing preconcentration with only one applied

8　　electric field (Figure 2B). Once the desired target analyte concentration has been achieved, as

9　　determined by fluorescence imaging of dye molecules conjugated to the analyte, the normal

10　　electric field is removed, and a tangential electric field moves the concentrated analyte by EOF

11　　through the suspended microchannel of the SMR, which is serially connected to the anodic

12　　microchannel of the NC (Figure 2C). The transient shift in resonant frequency of the SMR

13　　corresponding to the transit of the concentrated analyte is proportional to its density, and hence

14　　its concentration. This information, combined with the experimentally determined rate of

15　　preconcentration for a given analyte and the preconcentration time provides a quantitative

16　　measure of the analyte's initial concentration.

17

18　　3. Fabrication and Packaging

19

20　　A significant challenge in the integration of the fabrication processes for the standalone NC and

21　　SMR devices into a single process (Figure 3) was the requirement that the glass lid of the device,

1 which provides the upper surface of NC nanochannels, must not contain fluidic access holes.

2 The reason for this is that empirical studies have established that the presence of such holes

3 interferes with the anodic bonding of nanochannels in a way that is detrimental to the

4 performance of the NC (see Supplemental Material). Hence fluidic ports in standalone NC

5 devices have previously been made through the silicon base of the device. On the other hand,

6 fluidic ports on standalone SMR devices have exclusively been located in the glass lid because

7 the base of these devices contains glass frits which are required for vacuum packaging of the

8 resonators and are compromised by contact with fluids. [29] Therefore, in order to accommodate

9 the requirements of both the NC and SMR, a hybrid architecture was created in which part of the

10 device's lower surface is used for fluidic ports and the remainder used for resonator vacuum

11 packaging. In the completed integrated device (shown schematically in cross section in Figure

12 3J), the upper part of the package is provided by a continuous anodically-bonded glass lid. The

13 lower part of the package is a discontinuous frit-bonded glass base which hermetically encloses

14 the central portion of the device containing the resonator while leaving the fluid ports open to

15 external interconnects from below.

16

17 The front-side processing of the device (Figure 3A-G) was carried out in the Microsystems

18 Technology Laboratories at MIT. Starting substrates consisted of custom silicon-on-insulator

19 (SOI) wafers (150 mm-diameter, 675 µm total thickness, Icemos Technology, Belfast, UK)

20 containing 3 µm-deep channels buried within a 10 µm-thick silicon layer on top of a 2 µm-thick

21 silicon dioxide film (BOX). The buried channels define the suspended microchannel of the SMR,

1   and their surfaces possess a 500-nm thick thermally grown passivation oxide. Nanochannels

2   were etched into the silicon using a reactive ion etch (RIE) and their depth was found to be 42-43

3   nm using a contact profilometer. The anodic and cathodic microchannels of the NC were

4   defined by RIE, and the same etch was used to produce windows in the layer of silicon above the

5   suspended microchannel in order to enable fluorescence imaging of the contents of this buried

6   channel. Next, a series of deep reactive ion etches (DRIE) terminating on the BOX were used to

7   define the outline of the cantilever and the front-side portion of the device's fluidic ports. In

8   order to etch through the buried oxide film in the SOI device layer, the photolithographic pattern

9   corresponding to these features was etched a total of three times using two alternating DRIE

10   recipes selective to silicon and silicon dioxide. The silicon and silicon dioxide in the region of

11   overlap between the SMR and NC channels was removed by RIE, thus creating a connection

12   between them. A 500-nm thick wet thermal oxide was then grown on the wafers in order to

13   electrically insulate all exposed silicon surfaces. The BOX layer was removed from the bottoms

14   of the fluidic ports and the trenches surrounding the cantilevers by RIE. The SOI stack was then

15   anodically bonded to 500 µm-thick Pyrex wafers containing 50 µm-deep fluid channels and

16   resonator cavities which were produced using standard buffered oxide etching (BOE) procedures.

17   Anodic bonding conditions were empirically optimized together with the nanochannel

18   dimensions in order to achieve the highest possible bond strength without collapse of the

19   nanochannel (see Supplemental Material). These studies showed that a filter consisting of three

20   5 µm-wide nanochannels separated by 5 µm-wide pillars was the most resistant to nanochannel

21   collapse, and that this structure could be bonded with either a steel or graphite chuck at 400°C

22   and 975V with less than 10% of the bonded filters on the wafer displaying collapsed

1   nanochannels (n=15). This filter design was therefore employed in all integrated devices, and

2   wafer stacks were anodically bonded at 350°C and 1kV.

3

4   The back-side processing of wafer stacks (Figure 3H-J) was carried out by Innovative Micro

5   Technology (Santa Barbara, CA). Using the anodically-bonded glass lid as a handle, the bulk

6   silicon of the SOI stack was thinned to ~100 µm by mechanical grinding and chemical-

7   mechanical polishing. Cantilevers were released by a DRIE selective to silicon over silicon

8   dioxide which terminated on the BOX, and the same etch defined the bottom-side portion of the

9   fluidic ports. The SOI stack was then hermetically packaged under vacuum conditions by glass

10 frit bonding to a 500 µm-thick Pyrex wafer containing standoff structures to control the

11 compression of the frits and getters for gas sequestration. To prepare the Pyrex base for glass frit

12 bonding, standoffs were first produced by BOE, followed by silk screening of the glass frits, and

13 finally getter deposition. In order to enable the removal of Pyrex base material covering the

14 fluidic ports while maintaining the integrity of the resonator packaging, a novel glass frit design

15 and diesawing scheme was developed. As illustrated in Figure 3J, each device possessed two

16 continuous frits: an inner frit that encircled only the resonator, and an outer frit that followed the

17 perimeter of the die. Diesawing was carried out in two stages. First, partial-depth diesaw cuts

18 were performed on the Pyrex base just outside the inner frits, leaving a rectangular island of

19 Pyrex frit-bonded to the central resonator region of each device and connected to the rest of the

20 Pyrex base only by thin membranes. Next, full-depth diesaw cuts were performed just outside

21 the outer frits, separating the dies. The function of the outer frit is to prevent liquid from the full-

1   depth diesaw slurry from entering fluid ports, potentially contaminating device channels, or from

2   attacking the inner frits needed for resonator packaging. After diesawing, the sacrificial outer frit

3   was partially dissolved with acetone on individual devices to minimize the amount of mechanical

4   force needed to separate the eight pieces of peripheral Pyrex base material from the central island,

5   thus providing access to the device's fluid ports without damaging the vacuum-bearing inner frit.

6   Photographs of devices before and after removal of the peripheral Pyrex material are shown in

7   Figure 4.

8

9   4. Characterization

10

11   To establish the functionality of the device, a solution of bovine serum albumin (BSA)

12   conjugated to the fluorophore AlexaFluor 488 (Invitrogen, Carlsbad, CA), was concentrated

13   using the NC while monitoring its concentration by fluorescence imaging. After one minute of

14   preconcentration, the concentrated protein was flowed through the SMR while simultaneously

15   monitoring the SMR's resonance frequency. The fluorescence of the preconcentrated sample

16   just before transfer to the SMR could then be compared to the transient SMR resonance

17   frequency response during the sample transit in order to demonstrate the integrated SMR's

18   ability to quantify the amount of protein contained in the preconcentrated sample.

19

1   Further characterization was inhibited by the fact that integrated device packages were highly

2   fragile compared to those of standalone devices, so that fluidic interconnects previously

3   developed for simultaneous pressure and voltage control could not be employed. The increased

4   fragility of the integrated device arises from the fact that they are approximately half the

5   thickness of standalone devices in areas where sealing pressure is applied, because the bottom

6   Pyrex material must be removed from these areas. As a result, small bending moments that arise

7   from mechanical clamping of the fluidic interface can result in cleavage of the integrated devices.

8   Due to this limitation, fluids were introduced to the devices via reservoirs made from pipet tips

9   which were attached directly to the fluidic ports of the device using epoxy. Reservoirs were

10   filled with 15 mM phosphate buffered saline containing 142 nM AlexaFluor-BSA as target

11   analyte and 0.5 µm-diameter fluorescent polystyrene microspheres (Thermo Fisher Scientific,

12   Waltham, MA) at a concentration of $3x10^7$ beads/mL to visualize fluid flow patterns. Pressure-

13   driven fluid flow in the device was minimized by matching the fluid levels in the reservoirs.

14   Electric fields in the device were controlled by means of a DC power supply connected to

15   platinum electrodes contained in the reservoirs. Devices were mounted in a custom assembly

16   (shown schematically in Figure 5) designed to position the tip of the cantilever at the focus of a

17   laser for optical lever readout of its resonance frequency while mechanically actuating the device

18   using an external piezo crystal. Cantilever resonance is accomplished using a feedback

19   configuration as reported previously [29] with the addition of a limiter and a high-current output

20   stage to the feedback loop. These modifications were needed because of the piezo crystal's

21   greater capacitance at the resonance frequency relative to that of the electrostatic drive electrodes

22   of earlier devices. Devices were imaged using an upright epifluorescence microscope employing

1   a 120W metal halide lamp and a standard GFP filter set (Nikon Corp., Tokyo, Japan) coupled to

2   a Peltier-cooled CCD camera (Orca-ER, Hamamatsu Photonics K.K., Hamamatsu, Japan).

3   Images were analyzed using the commercial software package IPLab 3.7 (Scanalytics, Fairfax,

4   VA).

5

6   Experimental results for preconcentration and detection of AlexaFluor-BSA are summarized in

7   Figure 6, and movies of the experiments can be viewed online in the Supplemental Material.

8   Preconcentration was initiated by applying a potential difference of 50V between the anodic and

9   cathodic microchannels of the NC, and the resulting accumulation of fluorescent protein after a

10   period of one minute is shown in Figure 6B. The fluorescent intensity of the sample after one

11   minute of preconcentration varied considerably between experiments, indicating that the rate of

12   preconcentration was not constant, as with previously characterized standalone NC devices. To

13   quantify the rate of preconcentration, separate experiments were carried out where the anodic

14   microchannel was filled with AlexaFluor-BSA solutions having known concentrations, and the

15   resulting fluorescent intensity of the channel was recorded. Based on comparison of the

16   fluorescent intensities of preconcentrated samples to these standards, the average

17   preconcentration rate for the integrated NC was determined to be ~9000 ± 2000-fold/hour.

18   Following preconcentration, the potential difference between the anodic and cathodic

19   microchannels of the NC was removed, and a potential difference of 10-20V was subsequently

20   applied between the ends of the 1295 μm-long anodic microchannel, driving the concentrated

21   protein sample through the SMR by EOF (Figure 6C,D). Because the Debye length of 2.6 nm is

1   small compared to the channel width, EOF produces a relatively uniform flow profile, and

2   interactions with the channel walls are negligible, so the majority of the dispersion observed

3   during sample transfer can be attributed to diffusion. A typical transfer lasts less than ten

4   seconds, and the corresponding diffusion length of 35 μm for BSA [30] is negligible compared to

5   the 552 μm length of the suspended portion of the anodic microchannel; therefore, samples can

6   be transferred and detected before significant dilution takes place. Recorded SMR resonance

7   frequency time courses showed a transient decrease in the resonance frequency which

8   corresponds temporally to the transit of concentrated protein, as determined by the recorded

9   sequences of fluorescence images (Figure 7). The resonance frequency did not return to its

10   original baseline until several seconds after the data shown, which was attributed to the transit of

11   buffer ions that were locally concentrated by the NC. Since the concentrated protein sample

12   overlaps spatially with this region of increased ion concentration, the resonance frequency

13   minimum was compared to its baseline value immediately after the transit of protein to

14   determine the relative shift which was solely due to the presence of protein. This quantity was

15   found to be proportional to the measured fluorescence intensity of the protein sample just before

16   it was transferred to the SMR (inset of Figure 7), which indicates that the measured shift in

17   resonance frequency is an accurate measure of protein concentration under these experimental

18   conditions.

19

20   5. Conclusion

21

1   A device containing both NC and SMR structures has been fabricated using a novel packaging

2   process, and the integrated SMR has been used to measure the concentration of a protein which

3   was preconcentrated using the integrated NC. One obstacle that remains in the application of

4   this device to quantitative label-free sensing of analytes is the variability in the rate of

5   preconcentration observed with the integrated NC. This was attributed to differences in the

6   structure of the integrated NC's nanochannel filter compared to that of standalone NC devices.

7   Although the total cross-section of nanochannels, as determined by profilometry before anodic

8   bonding, was not significantly different between these two devices, the measured ionic

9   conductivity was almost an order of magnitude larger in the case of the integrated device. This

10   suggests partial delamination of the anodic bond, which may be due to changes in anodic

11   bonding conditions related to the modified channel layout. Weaker anodic bonds in integrated

12   devices could also result from a reduced electric field at the bond interface during bonding due to

13   the presence of additional silicon dioxide films in the SOI substrates which were not present in

14   substrates used to fabricate standalone NC devices. Another limitation of the increased filter

15   conductivity of the integrated NC is the high EOF through the filter, which diminishes

16   concentration polarization and causes the SCL to collapse against the filter for increasing values

17   of E_n. Since this situation can not be corrected by adding a tangential electric field, the

18   preconcentration rate is proportional to E_n^2, and the collapse of the SCL represents a practical

19   limitation on throughput. In contrast, standalone NC devices are able to support larger values of

20   E_n by increasing E_t, and since the preconcentration rate is proportional to the product of the two

21   electric fields in this mode of operation, a significantly higher throughput can be achieved.

22   Future integrated device designs should therefore be informed by more extensive studies on the

1   effects of local channel topography and substrate composition on the anodic bonding of

2   nanochannels in order to achieve the desired filter conductivity. The realization of such a device

3   will also make it possible to carry out preconcentration inside the SMR, which could open up

4   new possibilities for studying the spatial and temporal variations in ionic concentrations resulting

5   from preconcentration. In order to explore applications in immuno-detection and universal

6   detection, improvements in the fabrication and packaging processes are needed to make

7   integrated devices which are less fragile and hence compatible with pressurized fluid

8   interconnects required for active pressure-driven flow control. This will enable both site-specific

9   surface modifications, which are needed for surface immunoassays, and compatibility with

10   separation methods.

11

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