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Fabrication and Characterization of an Integrated Microsystem for
Protein Preconcentration and Sensing

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Abstract

We report on a fabrication and packaging process for a microsystem consisting of a mass-based protein detector and a fully integrated preconcentrator. Preconcentration of protein is achieved by means of a nanofluidic concentrator, which takes advantage of fast non-linear electro-osmotic flow near a nanochannel-microchannel junction to concentrate charged molecules inside a volume of fluid on the order of one picoliter. Detection of preconcentrated protein samples is accomplished by passing them through a suspended microchannel resonator, which is a hollow resonant cantilever serially connected to the nanofluidic concentrator on the same device. The transit of a preconcentrated sample produces a transient shift in the cantilever’s resonance frequency proportional to the density of the sample, and hence the concentration of protein contained in it. A device containing both nanofluidic concentrator and suspended microchannel resonator structures was produced using a novel fabrication process which simultaneously satisfies the separate packaging requirements of the two structures. Initial testing of this prototype device has demonstrated that the integrated suspended microchannel resonator can accurately measure the concentration of a bovine serum albumin sample which was preconcentrated using the integrated nanofluidic concentrator. Future improvements in the fabrication process will allow site-specific surface modification of the device and compatibility with separation methods, which will create opportunities for its application to immunoassays and universal detection.
1. Introduction

Recently, the Han research group developed a microdevice which takes advantage of fast non-linear electro-osmotic flow (EOF) in the vicinity of a nanochannel-microchannel junction to preconcentrate charged molecules by up to $10^4$-10$^6$-fold in less than an hour. [1, 2] This nanofluidic concentrator (NC) possesses several advantages over other biomolecular preconcentration techniques. Methods such as field-amplified sample stacking [3-6], isotachophoresis [7] and micellar electrokinetic sweeping [8-10] all have buffering requirements which can interfere with downstream sample processing, making them difficult to integrate with detectors. Electrokinetic trapping techniques [11-13] show promise as an efficient way to concentrate proteins, but the linearity and stability of these methods is a concern. Chromatographic preconcentration techniques [14-17] capture proteins by the hydrophobic interaction, and hence are biased toward large hydrophobic proteins and less sensitive to smaller or more hydrophilic molecules. Membrane filter preconcentration [18, 19] is another example of a method which is biased toward larger molecules. Compared to these alternatives, the NC method of biomolecular preconcentration is the most favorable in terms of general applicability to a wide range of molecular species, ease of integration, and rate of preconcentration. Existing NC devices with integrated sensing capabilities rely on fluorescent labeling of the target analyte molecules for detection. This approach is not ideal, because conjugation of target proteins to dye molecules can modify their chemical properties, which can interfere with sensing methods that require selective capture of the target by probe molecules. In addition, because fluorescent
detection is subject to photobleaching, autofluorescence of the sensing environment and
fluctuations in the excitation source intensity, quantitative sensing often depends on the
repeatability of calibration protocols.

These limitations can be overcome by integrating the NC with a suspended microchannel
resonator (SMR) sensor, as shown in Figure 1. The SMR is a resonant cantilever with a fluid-
filled microchannel running through it. The resonance frequency of the cantilever changes in
proportion to the buoyant mass of molecules and ions added to the suspended microchannel,
which makes it a general platform for quantitative, label-free detection in fluid. For example,
universal detection has been demonstrated by prefractionating mixtures by HPLC and measuring
the bulk density of the column’s output on an SMR. [20] The SMR has also been used to
conduct label-free immunoassays in blood serum by measuring the quantity of target protein
captured by antibody probes attached to the surfaces of the suspended microchannel. [21].

The sensitivity in both bulk density and surface based detection with the SMR would be
substantially enhanced by integrated preconcentration using a NC for sufficiently soluble protein
analytes. In addition, the SMR’s ability to measure the spatial and temporal dependence of ionic
concentrations in the vicinity of the NC may provide new information about the underlying
transport processes which could inform the design of future NC devices. Here we report a novel
fabrication process which was used to produce a microsystem containing both NC and SMR
structures. A monolithic system is required in order to allow the minute sample volume from the
preconcentrator to be transferred to the SMR without significant dispersion. Functionality of the
Integrated system has been demonstrated by preconcentrating a model aqueous protein solution using the NC, transferring the concentrated sample to the SMR, and measuring the final concentration obtained by SMR densitometry. By establishing successful operation of the NC and SMR simultaneously on the same device and quantitative transfer of preconcentrated protein samples between them, an important first step has been taken in the development of highly-sensitive universal detection systems and immunoassays.

2. Design

Integration of the NC and SMR was aided by the fact that the internal volume of existing standalone SMR devices (~25 pL), is comparable to the typical volume of concentrated protein samples which are produced in existing standalone NC devices (~1 pL) so that sample transfer and detection can be accomplished without significant dilution of the concentrated sample. Hence the physical dimensions of the NC and SMR components of the integrated device were made as similar as possible to those of standalone devices which have been previously demonstrated. [22, 1] The integrated NC structure consists of two microchannels with cross-sectional dimensions of 15 µm x 3 µm that are connected by a series of ~40 nm-deep nanochannels (Figure 2). When these nanochannels are filled with media having an ionic strength of ~10 mM, the Debye length is comparable with the nanochannel depth, which gives the nanochannel perm-selective ion transport properties when a longitudinal electric field, $E_n$, is applied. These nanochannels have silicon dioxide surfaces bearing negative fixed surface
charges at near-neutral pH, and hence positive counter-ions are transported more readily than negative co-ions. This results in depletion of counter-ions from the anodic end of the nanochannel and enrichment at the cathodic end. To maintain charge neutrality, co-ion concentrations mirror these changes in counter-ion concentration, producing a depletion region of reduced ionic strength at the anodic end of the nanochannel, a condition which is referred to as ion concentration polarization. [23] If $E_n$ is increased further, a situation may arise where the diffusion of counter-ions from the bulk will not be sufficient to maintain charge neutrality in the depletion region, in which case an extended space-charge layer (SCL) will form at the anodic end of the nanochannel. This layer can be thought of as an extension of the Debye layer of the nanochannel itself, with mobile counter-ions in the SCL screening fixed charges present on the nanochannel walls. The concentration of counter-ions in the SCL, which is linearly dependent on the magnitude of $E_n$, gives rise to non-linear electrokinetic phenomena when a tangential electric field, $E_t$, is applied longitudinally to the microchannel at the anodic end of the nanochannel. One of these phenomena is electro-osmotic flow (EOF) of the second kind [24, 25], which is proportional to the product of $E_n$ and $E_t$, and is much stronger than EOF observed ordinarily in microchannels, which scales simply as $E_t$. [26-28] Macromolecules are rapidly transported through the anodic microchannel by this non-linear EOF, and those possessing fixed charges with their own associated Debye layers are repelled from the depletion region, because the low ionic strength of this region makes it energetically unfavorable. This balance between strong EOF of the second kind and repulsion from the depletion region results in preconcentration of macromolecules at the outer boundary of the depletion region.
The main difference between the design of the integrated NC (Figure 2) and that of previous standalone NC devices [1] is the physical structure of the nanochannel filter. Although nanochannels in both cases were etched to the same depth of ~40nm, as determined by contact profilometry, differences in anodic bonding conditions between the two processes resulted in filters with higher ionic conductivities in the case of the integrated device. As a result, filters in the integrated device can support significant EOF with the application of a purely normal electric field. This makes it possible to achieve self-stabilizing preconcentration with only one applied electric field (Figure 2B). Once the desired target analyte concentration has been achieved, as determined by fluorescence imaging of dye molecules conjugated to the analyte, the normal electric field is removed, and a tangential electric field moves the concentrated analyte by EOF through the suspended microchannel of the SMR, which is serially connected to the anodic microchannel of the NC (Figure 2C). The transient shift in resonant frequency of the SMR corresponding to the transit of the concentrated analyte is proportional to its density, and hence its concentration. This information, combined with the experimentally determined rate of preconcentration for a given analyte and the preconcentration time provides a quantitative measure of the analyte’s initial concentration.

3. Fabrication and Packaging

A significant challenge in the integration of the fabrication processes for the standalone NC and SMR devices into a single process (Figure 3) was the requirement that the glass lid of the device,
which provides the upper surface of NC nanochannels, must not contain fluidic access holes. The reason for this is that empirical studies have established that the presence of such holes interferes with the anodic bonding of nanochannels in a way that is detrimental to the performance of the NC (see Supplemental Material). Hence fluidic ports in standalone NC devices have previously been made through the silicon base of the device. On the other hand, fluidic ports on standalone SMR devices have exclusively been located in the glass lid because the base of these devices contains glass frits which are required for vacuum packaging of the resonators and are compromised by contact with fluids. [29] Therefore, in order to accommodate the requirements of both the NC and SMR, a hybrid architecture was created in which part of the device’s lower surface is used for fluidic ports and the remainder used for resonator vacuum packaging. In the completed integrated device (shown schematically in cross section in Figure 3J), the upper part of the package is provided by a continuous anodically-bonded glass lid. The lower part of the package is a discontinuous frit-bonded glass base which hermetically encloses the central portion of the device containing the resonator while leaving the fluid ports open to external interconnects from below.

The front-side processing of the device (Figure 3A-G) was carried out in the Microsystems Technology Laboratories at MIT. Starting substrates consisted of custom silicon-on-insulator (SOI) wafers (150 mm-diameter, 675 µm total thickness, Icemos Technology, Belfast, UK) containing 3 µm-deep channels buried within a 10 µm-thick silicon layer on top of a 2 µm-thick silicon dioxide film (BOX). The buried channels define the suspended microchannel of the SMR,
and their surfaces possess a 500-nm thick thermally grown passivation oxide. Nanochannels were etched into the silicon using a reactive ion etch (RIE) and their depth was found to be 42-43 nm using a contact profilometer. The anodic and cathodic microchannels of the NC were defined by RIE, and the same etch was used to produce windows in the layer of silicon above the suspended microchannel in order to enable fluorescence imaging of the contents of this buried channel. Next, a series of deep reactive ion etches (DRIE) terminating on the BOX were used to define the outline of the cantilever and the front-side portion of the device’s fluidic ports. In order to etch through the buried oxide film in the SOI device layer, the photolithographic pattern corresponding to these features was etched a total of three times using two alternating DRIE recipes selective to silicon and silicon dioxide. The silicon and silicon dioxide in the region of overlap between the SMR and NC channels was removed by RIE, thus creating a connection between them. A 500-nm thick wet thermal oxide was then grown on the wafers in order to electrically insulate all exposed silicon surfaces. The BOX layer was removed from the bottoms of the fluidic ports and the trenches surrounding the cantilevers by RIE. The SOI stack was then anodically bonded to 500 µm-thick Pyrex wafers containing 50 µm-deep fluid channels and resonator cavities which were produced using standard buffered oxide etching (BOE) procedures. Anodic bonding conditions were empirically optimized together with the nanochannel dimensions in order to achieve the highest possible bond strength without collapse of the nanochannel (see Supplemental Material). These studies showed that a filter consisting of three 5 µm-wide nanochannels separated by 5 µm-wide pillars was the most resistant to nanochannel collapse, and that this structure could be bonded with either a steel or graphite chuck at 400°C and 975V with less than 10% of the bonded filters on the wafer displaying collapsed
nanochannels (n=15). This filter design was therefore employed in all integrated devices, and wafer stacks were anodically bonded at 350°C and 1kV.

The back-side processing of wafer stacks (Figure 3H-J) was carried out by Innovative Micro Technology (Santa Barbara, CA). Using the anodically-bonded glass lid as a handle, the bulk silicon of the SOI stack was thinned to ~100 µm by mechanical grinding and chemical-mechanical polishing. Cantilevers were released by a DRIE selective to silicon over silicon dioxide which terminated on the BOX, and the same etch defined the bottom-side portion of the fluidic ports. The SOI stack was then hermetically packaged under vacuum conditions by glass frit bonding to a 500 µm-thick Pyrex wafer containing standoff structures to control the compression of the frits and getters for gas sequestration. To prepare the Pyrex base for glass frit bonding, standoffs were first produced by BOE, followed by silk screening of the glass frits, and finally getter deposition. In order to enable the removal of Pyrex base material covering the fluidic ports while maintaining the integrity of the resonator packaging, a novel glass frit design and diesawing scheme was developed. As illustrated in Figure 3J, each device possessed two continuous frits: an inner frit that encircled only the resonator, and an outer frit that followed the perimeter of the die. Diesawing was carried out in two stages. First, partial-depth diesaw cuts were performed on the Pyrex base just outside the inner frits, leaving a rectangular island of Pyrex frit-bonded to the central resonator region of each device and connected to the rest of the Pyrex base only by thin membranes. Next, full-depth diesaw cuts were performed just outside the outer frits, separating the dies. The function of the outer frit is to prevent liquid from the full-
depth diesaw slurry from entering fluid ports, potentially contaminating device channels, or from attacking the inner frits needed for resonator packaging. After diesawing, the sacrificial outer frit was partially dissolved with acetone on individual devices to minimize the amount of mechanical force needed to separate the eight pieces of peripheral Pyrex base material from the central island, thus providing access to the device’s fluid ports without damaging the vacuum-bearing inner frit. Photographs of devices before and after removal of the peripheral Pyrex material are shown in Figure 4.

4. Characterization

To establish the functionality of the device, a solution of bovine serum albumin (BSA) conjugated to the fluorophore AlexaFluor 488 (Invitrogen, Carlsbad, CA), was concentrated using the NC while monitoring its concentration by fluorescence imaging. After one minute of preconcentration, the concentrated protein was flowed through the SMR while simultaneously monitoring the SMR’s resonance frequency. The fluorescence of the preconcentrated sample just before transfer to the SMR could then be compared to the transient SMR resonance frequency response during the sample transit in order to demonstrate the integrated SMR’s ability to quantify the amount of protein contained in the preconcentrated sample.
Further characterization was inhibited by the fact that integrated device packages were highly fragile compared to those of standalone devices, so that fluidic interconnects previously developed for simultaneous pressure and voltage control could not be employed. The increased fragility of the integrated device arises from the fact that they are approximately half the thickness of standalone devices in areas where sealing pressure is applied, because the bottom Pyrex material must be removed from these areas. As a result, small bending moments that arise from mechanical clamping of the fluidic interface can result in cleavage of the integrated devices. Due to this limitation, fluids were introduced to the devices via reservoirs made from pipet tips which were attached directly to the fluidic ports of the device using epoxy. Reservoirs were filled with 15 mM phosphate buffered saline containing 142 nM AlexaFluor-BSA as target analyte and 0.5 μm-diameter fluorescent polystyrene microspheres (Thermo Fisher Scientific, Waltham, MA) at a concentration of 3x10^7 beads/mL to visualize fluid flow patterns. Pressure-driven fluid flow in the device was minimized by matching the fluid levels in the reservoirs. Electric fields in the device were controlled by means of a DC power supply connected to platinum electrodes contained in the reservoirs. Devices were mounted in a custom assembly (shown schematically in Figure 5) designed to position the tip of the cantilever at the focus of a laser for optical lever readout of its resonance frequency while mechanically actuating the device using an external piezo crystal. Cantilever resonance is accomplished using a feedback configuration as reported previously [29] with the addition of a limiter and a high-current output stage to the feedback loop. These modifications were needed because of the piezo crystal’s greater capacitance at the resonance frequency relative to that of the electrostatic drive electrodes of earlier devices. Devices were imaged using an upright epifluorescence microscope employing
a 120W metal halide lamp and a standard GFP filter set (Nikon Corp., Tokyo, Japan) coupled to a Peltier-cooled CCD camera (Orca-ER, Hamamatsu Photonics K.K., Hamamatsu, Japan). Images were analyzed using the commercial software package IPLab 3.7 (Scanalytics, Fairfax, VA).

Experimental results for preconcentration and detection of AlexaFluor-BSA are summarized in Figure 6, and movies of the experiments can be viewed online in the Supplemental Material. Preconcentration was initiated by applying a potential difference of 50V between the anodic and cathodic microchannels of the NC, and the resulting accumulation of fluorescent protein after a period of one minute is shown in Figure 6B. The fluorescent intensity of the sample after one minute of preconcentration varied considerably between experiments, indicating that the rate of preconcentration was not constant, as with previously characterized standalone NC devices. To quantify the rate of preconcentration, separate experiments were carried out where the anodic microchannel was filled with AlexaFluor-BSA solutions having known concentrations, and the resulting fluorescent intensity of the channel was recorded. Based on comparison of the fluorescent intensities of preconcentrated samples to these standards, the average preconcentration rate for the integrated NC was determined to be \(~9000 \pm 2000\)-fold/hour. Following preconcentration, the potential difference between the anodic and cathodic microchannels of the NC was removed, and a potential difference of 10-20V was subsequently applied between the ends of the 1295 \(\mu\)m-long anodic microchannel, driving the concentrated protein sample through the SMR by EOF (Figure 6C,D). Because the Debye length of 2.6 nm is...
small compared to the channel width, EOF produces a relatively uniform flow profile, and interactions with the channel walls are negligible, so the majority of the dispersion observed during sample transfer can be attributed to diffusion. A typical transfer lasts less than ten seconds, and the corresponding diffusion length of 35 μm for BSA [30] is negligible compared to the 552 μm length of the suspended portion of the anodic microchannel; therefore, samples can be transferred and detected before significant dilution takes place. Recorded SMR resonance frequency time courses showed a transient decrease in the resonance frequency which corresponds temporally to the transit of concentrated protein, as determined by the recorded sequences of fluorescence images (Figure 7). The resonance frequency did not return to its original baseline until several seconds after the data shown, which was attributed to the transit of buffer ions that were locally concentrated by the NC. Since the concentrated protein sample overlaps spatially with this region of increased ion concentration, the resonance frequency minimum was compared to its baseline value immediately after the transit of protein to determine the relative shift which was solely due to the presence of protein. This quantity was found to be proportional to the measured fluorescence intensity of the protein sample just before it was transferred to the SMR (inset of Figure 7), which indicates that the measured shift in resonance frequency is an accurate measure of protein concentration under these experimental conditions.

5. Conclusion
A device containing both NC and SMR structures has been fabricated using a novel packaging process, and the integrated SMR has been used to measure the concentration of a protein which was preconcentrated using the integrated NC. One obstacle that remains in the application of this device to quantitative label-free sensing of analytes is the variability in the rate of preconcentration observed with the integrated NC. This was attributed to differences in the structure of the integrated NC’s nanochannel filter compared to that of standalone NC devices. Although the total cross-section of nanochannels, as determined by profilometry before anodic bonding, was not significantly different between these two devices, the measured ionic conductivity was almost an order of magnitude larger in the case of the integrated device. This suggests partial delamination of the anodic bond, which may be due to changes in anodic bonding conditions related to the modified channel layout. Weaker anodic bonds in integrated devices could also result from a reduced electric field at the bond interface during bonding due to the presence of additional silicon dioxide films in the SOI substrates which were not present in substrates used to fabricate standalone NC devices. Another limitation of the increased filter conductivity of the integrated NC is the high EOF through the filter, which diminishes concentration polarization and causes the SCL to collapse against the filter for increasing values of E_n. Since this situation can not be corrected by adding a tangential electric field, the preconcentration rate is proportional to E_n^2, and the collapse of the SCL represents a practical limitation on throughput. In contrast, standalone NC devices are able to support larger values of E_n by increasing E_t, and since the preconcentration rate is proportional to the product of the two electric fields in this mode of operation, a significantly higher throughput can be achieved. Future integrated device designs should therefore be informed by more extensive studies on the
effects of local channel topography and substrate composition on the anodic bonding of nanochannels in order to achieve the desired filter conductivity. The realization of such a device will also make it possible to carry out preconcentration inside the SMR, which could open up new possibilities for studying the spatial and temporal variations in ionic concentrations resulting from preconcentration. In order to explore applications in immuno-detection and universal detection, improvements in the fabrication and packaging processes are needed to make integrated devices which are less fragile and hence compatible with pressurized fluid interconnects required for active pressure-driven flow control. This will enable both site-specific surface modifications, which are needed for surface immunoassays, and compatibility with separation methods.


