Published in final edited form as:

Lab Chip. 2009 May 21; 9(10): 1429–1434. doi:10.1039/b818872f.

Electrical detection of protein biomarkers using bioactivated microfluidic channels

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Abstract

Current methods used for analyzing biomarkers involve expensive and time consuming techniques like the Sandwich ELISA which require lengthy incubation times, high reagent costs, and bulky optical equipment. We have developed a technique involving the use of a micro-channel with integrated electrodes, functionalized with receptors specific to target biomarkers. We have applied our biochip to the rapid electrical detection and quantification of target protein biomarkers using protein functionalized micro-channels. We successfully demonstrate detection of anti-hCG antibody, at a concentration of 1 ng ml⁻¹ and a dynamic range of three orders of magnitude, in less than one hour. We envision the use of this technique in a handheld device for multiplex high throughput analysis using an array of micro-channels for probing various protein biomarkers in clinically relevant samples such as human serum for cancer detection.

Introduction

In order to be able to diagnose diseases at an early stage, while the disease may still be curable, it is necessary that access be available to a sensitive platform which can rapidly and inexpensively detect and quantify a wide panel of biomarkers, biomolecular indicators signaling the presence or predisposition of a patient to a disease. There are various types of biomarkers, which if detected and quantified, can provide valuable information regarding the state of a disease. Examples of such are detection and isolation of particular rare cell types,¹ detecting genetic markers² associated with disease susceptibility, and also protein biomarkers.³ Protein biomarkers, such as various types of antigens and antibodies, present in the blood or serum are of particular interest. One example of a protein biomarker is the prostate specific antigen (PSA), which if found in the blood of males at levels greater than 4 ng ml⁻¹ can provide useful information for diagnosing prostate cancer.⁴ However, the test of PSA alone is insufficient in providing enough information to diagnose prostate cancer because of the heterogenous nature of the disease. The ability to detect and quantify a wide panel of biomarkers can effectively be used to diagnose diseases for any person within a population.

Current methods used for detection and quantification of protein biomarkers include techniques like the Sandwich ELISA,⁵ an expensive technique requiring bulky optical equipment and also labeling of the proteins. The long incubation times required (several hours) make this a rather time consuming technique. Proteins are also separated and recognized using Western blotting, based on gel electrophoresis, also requiring labeling of the proteins, making it an expensive and time consuming technique due to the reagent preparation required and the long separation time.⁶ Western blotting is typically used in conjunction with mass spectrometry to recognize and analyze proteins.⁷

In order to develop a platform which can be a good candidate for the clinical setting, it is desirable to have a rapid and inexpensive procedure which can be multiplexed to probe a complex biological sample for a wide array of biomarkers. Protein microarrays⁸ have also provided the ability for multiplexed quantification and detection. However, similar to all other fluorescence based detection techniques, protein microarrays require long incubation times and high reagent costs. The use of impedance based sensors for the detection of biomolecules^{9–19} eliminate the need for fluorescent labeling and also provide the opportunity for multiplexed analysis of biomolecules due to the ease of integrating CMOS (Complementary Metal Oxide Semiconductor), thus being a good candidate for the clinical setting. Nanogap sensors have been used to demonstrated detection of proteins.^{20–23} Using techniques such as resistive pulse sensing, changes in the size of functionalized microspheres have been used for demonstrating multiplexed target protein biomarker detection at concentrations as low as 15 ng ml⁻¹.^{24–26} With further optimization, it may be possible to decrease the detection limit by one or two orders of magnitude to achieve the detection limits required for cancer detection (4 ng ml⁻¹ for PSA). Capacitive electrical biosensors, based on changes induced by target molecule and probe binding on the surface charge on the electrode–electrolyte interface, have also been reported for detection of DNA hybridization and protein biomarkers.^{27,28} However, consistency in the results is problematic for these types of sensors. Recently, the electrical detection of protein biomarkers at detection limits as low as 1 pg ml⁻¹ has also been demonstrated using nanowires,²⁹ however the sensor operates at very low salt concentrations, making it incompatible with physiological conditions in clinical samples like blood. Nanowires are also more difficult and expensive to fabricate making it an unsuitable candidate for the clinical setting in the near future. Detection of protein biomarkers and nucleic acid biomarkers has been demonstrated at the single molecule level using solid-state nanopores.³⁰

Here we describe a chip-based microfluidic device capable of electrical real-time detection of target protein biomarkers in a multi-analyte solution. In a previous study we had demonstrated the ability of this technique to detect target cells without the need for any labeling.³¹ The purpose of this study was to investigate use of the described technique for specific protein biomarker detection. We have demonstrated the detection selectivity of this technique using anti-hCG antibody as our target protein biomarker. Anti-hCG antibody is a biomarker useful for diagnosing infertility in women if present in the serum at levels higher than 35 ng ml⁻¹.³² While we demonstrated the detection of anti-hCG antibody in this paper, we emphasize that this biomarker was chosen as an example, and that this technique can be applied generally to all protein biomarkers. Future studies will be directed towards improving the detection limit, multiplexed detection, and also detecting target biomarkers in clinical samples like blood and serum.

Protein biomarker detection with bioactivated micro-channel

In the micro-channel gating technique for protein biomarker detection, micron sized beads (Fig. 1a) are coated with primary receptors (Fig. 1b) and then the targeted protein biomarker is captured as the functionalized beads are immersed in a multi-analyte solution (Fig. 1c). Presented in Fig. 1d is the protein functionalized micro-channel biosensor, with gold electrodes labeled A and C. Protein receptors with affinities to target biomarkers are immobilized on the surface of the channel between electrodes A and C. The region between these two electrodes is the active region of the channel in which the ionic solution resistance is measured. Gold electrodes are very suitable for surface chemistry modifications, such as deposition of surface assembled monolayers, which will optimize the immobilization of the receptors. The beads are then injected into the micro-channel (Fig. 1e) partially occluding the channel resulting in a resistance higher than the baseline value. If any of the bead surfaces are labeled with the targeted biomarkers, the beads will attach to the receptors on the channel wall. After the beads have come to rest, a flow is applied across the channel causing the unbound beads to be washed out

of the channel, resulting in a drop in the ionic solution resistance depending on the number of beads remaining (Fig. 1g). The number of beads remaining attached is proportional to the targeted protein biomarker concentration. A high concentration of target biomarkers will result in a smaller drop in resistance compared to a low concentration of biomarkers. Thus, in addition to being able to detect the presence of protein biomarkers at low concentration, this sensor also provides the ability of measuring the concentration of the target biomarker.

The requirement for successful detection of the target biomarker is that the surfaces of the microspheres contain primary receptors and that the active area of the sensor contains secondary receptors, both of which should be specific to the targeted biomarker. It is also necessary that the microspheres used be comparable in size to that of the channel geometry.

Electrode electrolyte interface

The physical processes occurring at the interface between the electrode and the electrolyte and also the bulk solution directly dictate the impedance behavior of the channel.¹⁷ Fig. 2a shows a side view cross section of the device. The gold electrode surfaces are assumed to be hydrophilic. An equivalent circuit network (Fig. 2b) based on the parasitic resistances and capacitances between electrodes A and C (electrode B is floating) can be used to describe the impedance behavior. The small separation of the layer of accumulated ions (Fig. 2a) on electrodes A and C results in the double layer capacitance, $C_{\text{double layer}}$ which is measured to be approximately 0.4 nF for our system, dominating the impedance at low frequencies.¹⁶ Effects such as the Warburg impedance and the electron transfer resistance also significantly effect the impedance at low frequencies.³³ We did not include either of these two parasitic impedances in our equivalent circuit model since they are negligible at the frequencies which we operate. Due to the large separation, the capacitance between the electrodes A and C, C_{cell} , is negligible. Given that we want to detect the presence of the microspheres due to the resulting change in channel resistance, we want to minimize the effect on the impedance measurement resulting from all impedances except for the bulk solution resistance, R_{solution} . This can be achieved by working at sufficiently high frequencies. From our previous work,³¹ we measured the onset of bulk solution resistance dominating the impedance at frequencies above 20 KHz. Bulk solution resistance levels were measured to be approximately 80 K Ω with a salt concentration of 138 mM NaCl. We found approximately 30 KHz to be an optimum frequency to operate our device, because at frequencies below this, the impedance due to the double layer capacitance had not yet completely diminished, however as the frequency of the excitation voltage signal was increased above 30 KHz, the output signal became noisier.

Methods and materials

In this section we discuss the design of the biomicrofluidic chip, the fabrication of the micro-channel and the electrodes on the glass substrate, the measurement instrumentation for monitoring the current across the electrodes, the coating of the beads with antibodies, the bioactivation of the surface, and the detection assay of the anti-hCG.

Device design

The microfluidic biochip used in this study is shown in Fig. 3a. Experiments were conducted on micro-channels of various sizes 10 μm deep and 20 μm wide, and 50 μm deep and 50 μm wide channels (Fig. 3c). For smaller channel sizes, channel clogging and nonspecific binding was problematic, so the larger channel sizes were used.

Fabrication

Au/Cr electrodes (2000 Å/150 Å) were micropatterned on a glass wafer using traditional photolithography, sputtering, and then lift-off processing and then cut into separate chips using

a wafer saw. The micro-channels were fabricated in PDMS (fabricated by the Stanford Microfluidics Foundry).

The master mold for the micro-channels was patterned onto a silicon substrate using SU-8 photoresist. PDMS (10 : 1 pre-polymer : curing agent) was poured onto the master mold and allowed to cure. The glass chips and the PDMS slabs were aligned and then bonded together after oxygen plasma treatment.

Measurement apparatus

Electrical impedance measurements were collected across the channel in the region between electrodes A and C. We applied a voltage signal to electrode A and a low noise current pre-amplifier (Stanford Research Systems Model SR570) to electrode C in order to measure the current across the channel and then the data was collected with a National Instruments data acquisition card and read by a Labview program. The channels were also monitored using optical microscopy in order to confirm that the electrical signal changes were due to beads binding inbetween the electrodes.

Microsphere preparation

Anti-rabbit IgG, which has a specific affinity to rabbit anti-hCG antibody, was used as the primary receptor which was physically adsorbed onto 10 μm polystyrene beads (Bangs Labs, WI). The microspheres were suspended in 50 μl of PBS buffer at a concentration of 11.8 mg ml^{-1} . 10 μl of anti-rabbit IgG (Sigma Aldrich, St. Louis, MO), at a concentration of 5 $\mu\text{g ml}^{-1}$, was added to the bead solution, and incubated in a rotator for 45 min in order to prevent precipitation. The solution was then centrifuged, the supernatant was removed, and the beads were again resuspended in PBS. This process was repeated three times in order to ensure that all free antibodies were removed from the solution.

Channel surface bioactivation

Anti-rabbit IgG was also used as the secondary receptor which was physically adsorbed onto the base of the microfluidic channel. Anti-rabbit IgG diluted in PBS solution to 5 $\mu\text{g ml}^{-1}$ was injected into the channel and incubated for 15 min. The micro-channel surface was then coated with a blocking buffer, 1 mg ml^{-1} bovine serum albumin (BSA) in order to minimize nonspecific interactions. BSA solution was injected into the channel and incubated for 10 min. Since the probe molecules could be physically adsorbed onto the glass base of the channel, the use of a channel with a wide floating electrode (electrode B) inbetween the active electrodes (A and C) was unnecessary. Future surface chemistry optimization will be performed on channels with a gold region inbetween the active electrodes.

Anti-hCG antibody assay

For the test sample, PBS solution was spiked with various concentrations of anti-hCG antibody ranging from 10 pg ml^{-1} to 1 $\mu\text{g ml}^{-1}$. The functionalized beads were immersed in the test sample, and placed in a rotator for 45 min to capture target proteins in the sample. Anti-rabbit IgG molecules capture anti-hCG antibodies based on an interaction which occurs between the Fab region of the anti-rabbit IgG and an epitope located on the Fc region of the anti-hCG antibody. The solution was then centrifuged, then resuspended in PBS. This process was repeated three times in order to ensure that the free target protein molecules were removed completely from the solution.

The bead solution was injected into the micro-channel and incubated for 1 min to allow the beads which captured the target protein to bind to the base of the channel forming a sandwich assay. Fluids were injected into the channel, and the flow rates were controlled using a pressure

driven Harvard Apparatus Model 11 syringe pump (Instech Solomon, Plymouth Meeting, PA). A flow rate of 50 nl min^{-1} , which was experimentally determined to be the minimum flow rate required to wash off the nonspecifically bound beads, was then applied to the micro-channel in order to flush out the unbound beads. The number of beads before and after the washing was counted manually, and the electrical impedance was recorded simultaneously.

Results and discussion

In order to demonstrate the ability of our technique to detect the target biomarker, we performed real-time electrical measurements. We looked at the percentage drop in resistance across the channel after washing. The percent change provides information as to how many beads are removed from the channel as compared to how many were present before the washing step. The electrical measurements are shown in real-time (Fig. 4a) as the channel was washed. As the flow was applied to the channel, the unbound beads are flushed out of the channel. As the concentration of the target protein biomarker decreases, the drop in the electrical impedance increases. The decrease in the target biomarker concentration results in more beads being removed from the sensing area of the channel (Fig 4a), thus resulting in a larger drop in impedance across the electrodes. When the target concentration is $1 \mu\text{g ml}^{-1}$, almost all of the beads remain attached (Fig. 4b) corresponding to no change in the impedance after washing. In the scenario where no target protein was present in the test sample, almost all of the beads were removed from the base of the channel, with the exception of a few which remain attached due to nonspecific binding. This corresponds to the largest drop in impedance (Fig. 4c).

We analyzed the ability of this technique to quantify target protein biomarkers in detail by performing this assay over a wide range of target protein concentrations. The assay was confirmed optically (Fig. 5a), where the beads in the channel were counted before and after washing. The standard error bars for over five different experiments for each data point is included. A dynamic range of three orders of magnitude and a repeatable detection limit of 1 ng ml^{-1} (7 pM) are demonstrated. The average percentage decrease in electrical resistance measured as a function of target biomarker concentration is shown in (Fig. 5b) confirming the optical results (Fig. 5a). Decrease in target biomarker concentration results in more beads being removed from the sensing area of the channel, thus resulting in a larger drop in resistance across the electrodes. As a control experiment, we tested the case where no target protein biomarkers were present in the test sample, which resulted in an average capture of less than 4 % of the beads due to nonspecific binding of the beads, and a 0.5 % drop in impedance.

The standard error bars for the electrical measurements are greater than the standard error bars for the optical measurements. The impedance sensitivity to the location of the beads between the electrodes is the main cause for this inconsistency. The current method used for solving this problem is to run the experiment at least five times and to plot the average value as shown in Fig. 5b. This results in a higher resolution of biomarker concentration, making quantification using this technique practical. There are several other methods for reducing the standard error bar for the electrical quantification measurements, which we are currently exploring. One possibility is to integrate interdigitated electrodes at the base of the channel across the whole channel, effectively increasing the active area of the sensor. Another possibility is to integrate multiple sets of electrodes across the whole channel, which will not only effectively increase the active area of the sensor, it will also have a higher electrical sensitivity than a channel with interdigitated electrodes.

Although we performed the capture of the target protein biomarker off-chip, it is also possible to perform this step on-chip to inject the test sample in the microfluidic channel so that the target proteins get captured by the primary receptors immobilized on the surface of the channel.

Doing so would simplify the process for preparing reagents and remove the need for the centrifuging step required in order to remove the free target proteins from the solution.

One of the main advantages of our techniques is that it is able to operate at salt concentrations as high as 138 mM NaCl or even 800 mM NaCl, as opposed to other techniques such as the use of bionanoFETs where they are limited to salt concentrations as low as 2 μ M,²⁹ which makes them unable to operate in physiologically relevant samples. This opens the door to the possibility of direct detection of biomarkers in clinical samples such as blood or serum.

We describe here a method for electrically detecting target protein biomarkers without the need for labeling and bulky readout instruments. We have achieved a detection limit comparable to sandwich ELISA, which tends to achieve detection limits above 10 pM.³⁴ Electrical detection is much more inexpensive and can be easily multiplexed and integrated into a portable device useful for analyzing a wide panel of markers. Electrical detection also eliminates the need for fluorescent labeling which lowers the costs of the reagents.

While we demonstrated the detection of anti-hCG, we emphasize that this assay is applicable to all protein biomarkers. Our technique has the advantage of real-time, electrical detection and quantification of target biomarkers. By fabricating multiple channels onto a single chip and immobilizing different antibodies in each of the channels, this technique can be used for multiplex sensitive high throughput analysis for probing a complex mixture, which is of utmost necessity for point-of-care and early stage diagnosis.

Conclusions

Our technique addresses the clinical need for developing an inexpensive platform for analyzing a wide panel of biomarkers necessary for early disease diagnosis. In this paper we present a device using a functionalized micro-channel with integrated electrodes which can be used for multianalyte detection and quantification. We have demonstrated the detection of a target biomarker with a detection limit of 1 ng ml⁻¹ and a dynamic range of three orders of magnitude, and the ability to operate at high salt concentrations. We have described the design, fabrication, and the electronic apparatus employed for testing our sensors in detail. Given that our technology has the ability to be multiplexed, we envision that this technique opens many new doors in the development of high throughput devices used in the clinical setting.

Acknowledgments

This research was supported by National Institutes of Health Grant P01 HG000205. The authors would like to thank Jessica Melin and the Stanford Microfluidics Foundry for their invaluable help in fabrication of the devices used in this study.

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Fig. 1.

(a) Micron sized bead. (b) Bead coated with receptors and then (c) immersed in a multi-analyte solution. (d) Protein functionalized micro-channel biosensor. (e) The beads labeled with targeted biomarkers, in a phosphate buffered saline (PBS) solution (138 mM NaCl, 2.4 mM KCl) with a pH of 7.4, are loaded into the channel and allowed to bind to the secondary receptor molecules which are immobilized on the gold electrode forming a (f) sandwich assay at the channel surface (top plot). (Bottom plot) Prediction of resistance across electrodes A and C after injection of beads. (g) The channel is then flushed, causing the unbound beads to be removed from the channel. The magnitude of the resistance change across electrodes A and C is proportional to the target biomarker concentration.

Fig. 2.

(a) Side view cross section of microfluidic sensor. Positive and negative circles represent positive and negative ions accumulating at the surface of electrodes A and C. Electrode B is floating. A function generator is tied to the left electrode and a current pre-amplifier is tied to the right electrode, which is used to measure the current across the channel. (b) An equivalent circuit used in our system for understanding the impedance behavior as a function of frequency.

Fig. 3.
 (a) Schematic of microfluidic biochip. PDMS chip and glass chip bonded together. (b) Photograph of a single chip containing three different channels with integrated electrodes. (c) Optical image of 50 μm channel with three electrodes. Resistance is measured between electrodes A and C. Electrode B is not used in this study.

Fig. 4.

(a) Percentage change in resistance as a function of time. The real time drop in resistance increases as the concentration of target protein biomarker decreases. (b) High concentration of target protein results in almost all beads remaining bound after washing. (c) No target protein in test sample results in almost all beads being washed off.

Fig. 5.

(a) The percentage of beads remaining attached in the micro-channel after incubation, with different concentrations of target protein biomarker as measured optically, establishing a dynamic range of 3 orders of magnitude. A detection limit of 1 ng ml^{-1} has been demonstrated.

(b) The percentage decrease in ionic impedance across the channel as a function of protein biomarker concentration with standard error bars. Detection limit of 1 ng ml^{-1} and dynamic range of three orders of magnitude demonstrated.