Biomarker Analysis on a Power-free Microfluidic Chip Driven by Degassed Poly(dimethylsiloxane)

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Point-of-care testing (POCT) of biomarkers, such as proteins and nucleic acids, is a hot topic in modern medical engineering toward the early diagnosis of various diseases including cancer. Although microfluidic chips show great promise as a new platform for POCT, external pumps and valves for driving those chips have hindered the realization of POCT on the chips. To eliminate the need for pumps and valves, a power-free microfluidic pumping method utilizing degassed poly(dimethylsiloxane) (PDMS) was invented in 2004. In this article, the working principle of the degas-driven power-free microfluidic chip is first described, and then applications of those chips to biomarker analysis are reviewed. The biomarker analysis on the chip was typically achieved with a small sample volume of ∼1 μL and a short analysis time of ∼20 min. For protein analysis, the sandwich immunoassay format was adopted. The limit of detection (LOD) was improved by three orders of magnitude by using laminar flow-assisted dendritic amplification (LFDA), which was a newly devised amplification method specialized for microfluidic chips. For analysis of nucleic acids such as DNA and microRNA, the sandwich hybridization format was adopted, and the LFDA was also effective to reduce the LOD. With the LFDA, typical LOD values for proteins and nucleic acids were both around 1 pM.

Keywords Biomarker, microfluidic chip, microRNA, power-free, degas-driven, point-of-care testing

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1 Introduction

A biomarker is a measurable characteristics that reflects the presence or severity of a disease state. This broad definition of biomarkers includes traditional physiological parameters, such as body temperature and blood pressure. However, nowadays, the definition of biomarkers is practically limited to chemical substances in body fluids. Especially, most of the established biomarkers are proteins in blood. As described in Sect. 3.1, C-reactive protein (CRP) and prostate-specific antigen (PSA) are typical examples of those protein biomarkers. Recently, nucleic acids in blood have also been intensively studied as new biomarker candidates. For example, microRNAs (miRNAs) in blood are very promising as cancer biomarkers, as described in Sect. 4.2.

While the discovery of biomarkers has been of great interest in medical research, the analysis of biomarkers is also becoming a hot target of technological development. There has already been a "gold standard" analysis technology for every biomarker species: immunoassays for proteins and quantitative polymerase chain reaction for nucleic acids. Although these technologies are best suited in most situations at present, there remains much room for improvement. Common drawbacks of these technologies are long analysis time and bulky equipment. These
technologies are basically designed for use in centralized testing laboratories. In contrast, testing in a more decentralized manner, namely point-of-care testing (POCT), has been of great interest in modern medical engineering. More specifically, POCT is defined as diagnostic testing at, or near, the point of patient care. Successful POCT devices include blood glucose sensors and pregnancy test strips. They require minimal analysis time and equipment. However, they cannot be straightforwardly applied to other biomarkers, because of the much lower concentrations of those biomarkers in body fluids.

Recently, microfluidic chips have shown great promise as a new platform for high-performance POCT. A microfluidic chip is a centimeter-scale plate containing microchannels for fluid transport. The microchannels have typical cross-sectional dimensions of tens to hundreds of micrometers. Research on microfluidic chips in the community of analytical chemistry has a long history, since the 1990’s. The downsizing of the space of chemical reaction has an acceleration effect in some cases. A representable example is solid-phase immunoassay, which takes several hours in a conventional scale because of the slow, diffusion-limited mass transport. This process can be dramatically accelerated by the use of a microfluidic chip because of the scaling law of diffusion. Specifically, the time for diffusion is proportional to the square of the characteristic length. This means that 100-fold downsizing, which is achieved by a typical microfluidic chip, leads to a 10000-fold reduction in the diffusion time. Therefore, on a microfluidic chip, diffusion is no longer the rate-limiting process in an immunoassay, which is typically completed in 20–30 min without spoiling the sensitivity or specificity.

Although the use of microfluidic chips for POCT has a great advantage in terms of the analysis time, it raises a new technical challenge: how to introduce the fluid into the microchannel. Most microfluidic chips still depend on external pumps and valves. These mechanical devices are difficult to reduce in terms of footprint and cost, and therefore have hindered the realization of POCT with microfluidic chips. To eliminate the need for those mechanical devices, a few microfluidic pumping methods without external power sources have been reported. These “power-free” pumping methods were based on different principles, such as capillary action and gravity, with their own advantages and drawbacks. In 2004, our group reported a novel principle for power-free microfluidic pumping: fluid pumping by degassing and re-absorption processes are governed by time-reversible equations. This lifetime can be extended by covering the upper face of the PDMS piece, and seems sufficient for most of POCT applications.

2 Power-free Microfluidic Pumping by Degassed PDMS

Figure 1 shows the simplest type of microfluidic chip that our group has been using. The microfluidic chip has four straight microchannels (the solid lines in Fig. 1A). Each microchannel is connected to an outlet waste reservoir with a diameter of 2.5 mm. A cross-sectional view of the microfluidic chip is shown in Fig. 1B (only one microchannel is shown for simplicity). The PDMS part has grooves with a width of 100 μm and a depth of 25 μm. The grooves become microchannels by bonding the PDMS part with the flat glass plate.

PDMS is a common type of silicone elastomer, and is the most popular material for microfluidic chips because of its easy fabrication, high chemical stability, and high optical transparency. PDMS has another unique property: high gas solubility, which was exploited for power-free microfluidic pumping by our group for the first time. In fact, solidified PDMS dissolves a large amount of gas. At atmospheric pressure, the solubility of air is about 0.1 volume per 1 volume of PDMS. The gas solubility obeys Henry’s law, similar to the solubility in liquids. Namely, it is proportional to the gas pressure around the PDMS. Therefore, if we put a PDMS piece in a vacuum, the air inside the PDMS piece is removed. If we bring the “degassed” PDMS piece back to the atmosphere, it absorbs air again. This re-absorption action can be utilized for the microfluidic liquid pumping, as we will describe in the next two paragraphs.

In other words, the energy for the liquid pumping is stored in the PDMS piece as a form of chemical potential: un-equilibrium in gas concentration. The time required for the degassing and re-absorption is dependent on the size of the PDMS piece. In the case of an ordinary microfluidic chip with a millimeter to centimeter scale, the gas transport phenomena would take several ten minutes. More specifically, our calculation clarified that 99% degassing would be completed within 40 min for a PDMS piece of 2 mm thickness. This means that the lifetime of the degassed PDMS is also around 40 min, because the degassing and re-absorption processes are governed by time-reversible equations. This lifetime can be extended by covering the upper face of the PDMS piece, and seems sufficient for most of POCT applications.

The operation of the power-free microfluidic chip is depicted in Fig. 2. First, the microfluidic chip is put in a vacuum chamber, and degassed for at least 40 min. Immediately after the microfluidic chip is taken out from the vacuum chamber, a piece of adhesive tape is attached onto the PDMS part so that the outlet waste reservoirs are closed. In our group’s case, the microfluidic chip is usually used for the analytical experiment soon (in 1–5 min). However, it can also be stored in a vacuum container for later use. Therefore, the degassing step can be done separately from the analytical experiment. To start the analytical experiment, the user simply applies the liquid to be pumped, typically ~1 μL in volume, using an ordinary micropipette.

The mechanism of the power-free microfluidic pumping is depicted in Fig. 3. The air inside the microchannel and the waste reservoir dissolves into the PDMS part through the walls, because the PDMS was degassed in advance. When the inlet of the microchannel is blocked with the pipetted droplet, air is no longer supplied from the outside, because the outlet was already blocked with the adhesive tape. In this case, the air dissolution

![Fig. 1 Typical power-free microfluidic chip. (A) Photograph of the chip. A piece of adhesive tape was attached onto the chip to cover the outlet waste reservoirs. (B) Simplified cross-sectional view of the chip.](image-url)
into the PDMS reduces the pressure inside the microchannel-reservoir system. As a result, the pressure reduction draws the liquid from the inlet. Even after the microchannel is filled with the liquid, the pumping is continued by the air dissolution in the reservoir until the reservoir is completely filled with the liquid. This point is the largest difference between the two passive pumping methods: the degas-driven method (described above) and the capillary action method. Namely, in the capillary action method, the pumping basically stops when the narrow microchannel part is filled with the liquid. Although further continuation of the capillary action pumping is possible with some additional structure, e.g., an evaporator at the end of the microchannel,10 this is a significant limitation for application of the capillary action method. A typical flow rate of deionized water driven by the degassed PDMS through the microfluidic chip shown in Fig. 1 was 3 – 5 nL/s (linear flow rate of 1 – 2 mm/s).8 This means that a 0.5 μL aliquot, a typical aliquot size for applications described below, takes 1.5 – 3 min to flow through the microchannel.

### Application to Protein Analysis

#### 3-1 Fluorescence immunoassay without amplification

Immunoassay is currently the predominant analytical technology in which the specific interaction of an antibody with its antigen is exploited for molecular recognition. Especially, an immunoassay is one of the standard methods for detection and quantification of biomarker proteins. For example, an immunoassay is routinely used for measuring the CRP in human serum as a biomarker of various diseases including autoimmune diseases, malignancy, and cardiovascular diseases.11 Another example relevant to the current article is PSA in male human serum as a biomarker of prostate cancer.12 These biomarker proteins are usually measured by the solid-phase, sandwich-type immunoassay depicted in Fig. 4. In this type of immunoassay, two different antibodies, which are both specific to the target biomarker protein, are used. One antibody (primary antibody) is immobilized onto a solid surface, such as the wall of a container or microparticle surfaces. When the solid surface is reacted with the sample, the target molecule in the sample is captured by the immobilized primary antibody molecule. After that, the solid surface is reacted with the solution of the other antibody (secondary antibody), which is labeled with various molecules, such as fluorescent dye (fluorescence immunoassay, FIA) or enzyme (enzyme-linked immunosorbent assay, ELISA). The amount of the labeling molecules on the solid surface is measured to estimate the amount of the target molecules in the
sample. The solid surface enables easy separation of the bound molecules from the free molecules.

As the first trial of the application of the power-free microfluidic chip to protein detection, FIA of CRP was tried. After degassing the microfluidic chip shown in Fig. 1, the following solutions were sequentially injected into one of the microchannels. (1) 0.5 μL of anti-CRP antibody solution. The antibody molecules are considered to have been physically adsorbed onto the PDMS surface, because PDMS surface is hydrophobic and tends to adsorb protein molecules. (2) 0.5 μL of bovine serum albumin (BSA) solution for blocking purpose. (3) 1.0 μL of sample solution containing various concentration of CRP. (4) 0.5 μL of fluorescein-labeled anti-CRP solution. (5) The microchannel was washed with a flow of 0.5 μL of a BSA solution twice. The total time required for steps (1) through (5) was about 20 min. Next, the microchannel was observed using a fluorescence microscope, and the fluorescence intensity was measured from the image. As expected, the fluorescence intensity increased with increasing concentration of CRP in the sample. This means that the FIA of CRP was successful with a very short analysis time of 20 min and an extremely small sample volume of 1.0 μL. However, the obtained limit of detection (LOD), 0.42 nM, was not very impressive. Although this LOD reached the standard of immunoassays on microfluidic chips at the time, it was worse than the macroscale ELISA by two orders of magnitude. Improvement of the LOD was the next agenda.

3-2 Laminar flow-assisted dendritic amplification

The essential difference between the above-mentioned microfluidic FIA and the conventional ELISA was evident. While the microfluidic FIA included no amplification step, the conventional ELISA includes an enzymatic amplification step, in which a single enzyme molecule conjugated with the secondary antibody converts thousands of the substrate molecules into detectable product molecules. In other words, the ELISA produces a thousands of times more intense signal than the FIA under the same condition. However, to our experience, implementation of the enzymatic amplification in the microfluidic chip had some technical difficulties, because the number of reaction steps increased from 5 to 10. This would be inconvenient as a POCT method.

To reduce the number of steps, a new DA method, laminar flow-assisted dendritic amplification (LFDA, Fig. 5B) was devised. Laminar flow, which means fluid flow without convection, is a common phenomenon in microchannels because of the low Reynolds numbers. Therefore, when two different solutions are pumped into a microchannel, they are mixed only by diffusion through the interface between the two streams. At the contact line among the two streams and the channel ceiling (or the channel floor), the components in the two streams are simultaneously and continuously supplied onto the solid surface. In the current example, the components are F-SAs and B-anti-SA. They are supplied to the sandwich immunocomplex on the PDMS channel ceiling using a Y-shaped microchannel.

The LFDA method was applied to the immunoassay of CRP on the power-free microfluidic chip. After immobilization of the primary antibody and blocking in a similar way as the ordinary DA method described above, 0.5 μL of a CRP sample solution was injected from the left inlet of the Y-shaped microchannel, while 0.5 μL of a B-anti-CRP solution was injected from the right inlet. Next, 3.0 μL of F-SA was injected from the left inlet, while 3.0 μL of B-anti-SA was injected from the right inlet. The fluorescence intensity of the microchannel was measured in real time. As expected, the center of the microchannel showed strong fluorescence, which grew of the microfluidic chip shown in Fig. 1, the following solutions were sequentially injected into one of the microchannels. (1) 0.5 μL of anti-CRP antibody solution. (2) 0.5 μL of blocking solution. (3) 1.0 μL of sample solution. (4) 0.5 μL of fluorescein-labeled anti-CRP solution. (5) 0.5 μL of F-SA solution. (6) 0.5 μL of B-anti-SA solution. Next, steps (5) and (6) were further repeated twice to make 3-layer dendritic structures. As a result, the fluorescence intensity increased with increasing CRP concentration and increasing layer number (n in Fig. 5A). With 3 layers, the LOD became 0.21 pM, which means improvement from the previous work (0.42 nM) by 3 orders of magnitude. The DA turned out to be very effective for the microfluidic immunoassay. However, the number of reaction steps increased from 5 to 10. This would be inconvenient as a POCT method.

Fig. 5 Schematic representation of the two modes of DA for sandwich immunoassay. (A) The conventional DA. (B) The LFDA. Adapted from Ref. 15 with permission.
4 Application to Nucleic Acid Analysis

4.1 DNA

DNA analysis plays a crucial role in almost every field of modern life science and technology. One of the most common technologies for DNA analysis is hybridization on solid-phase supports, which forms the principle of microarrays. Although the microarray technology enables massively parallel DNA analysis, it takes a long incubation time of several hours to overnight. The reason for the long incubation time is the same as the solid-phase immunoassays: diffusion-limited mass transport. This problem can be solved by using microfluidic chips. However, most of the previous microfluidic chips required external apparatuses for fluid pumping, which raised complexity and cost of the whole system.

The combination of the degas-driven power-free microfluidic pumping and LFDA was applied to DNA analysis. As shown in Fig. 6A, a double-Y microchannel layout was adopted instead of the simple Y-shaped microchannel layout used for the immunoassays. This is because the main purpose of this work was to identify single-nucleotide polymorphism (SNP), and the simultaneous detection of two possible alleles was important in this case. The two halves of the double-Y microchannels were not completely independent; the two channels at the center were always used for the same solution. The capture probe (CP) DNA was covalently immobilized onto the glass surface by the epoxy-amine coupling method. After immobilization, the PDMS part with the microchannel pattern was reversibly bonded onto the glass surface so that the microchannels overlapped the CP spot. Alignment was achieved by adjusting the fabricated pattern on the PDMS part and a visible mark on the glass plate made by a marking pen. The two parts were bonded just by attaching them to each other.

First, we evaluated the sensitivity and specificity of DNA detection by this method using a biotinylated target DNA of 21 bases (B-tar in Fig. 6). In this case, the left half of the double-Y microchannels was used for various concentrations of B-tar, while the right half was used for blank reference. All the samples and the blank reference contained excess amount of biotinylated DNA with a random 21-base sequence. After degassing of the microfluidic chip, the blocking solutions were injected from the left inlet (0.5 μL), the right inlet (0.5 μL), and the center inlets (1.0 μL). Next, the B-tar sample and the blank were injected from the left and right inlets, respectively (0.5 μL each), while the blocking solution was injected from the center inlets (1.0 μL). Finally, LFDA was started by injecting B-anti-SA solutions from the left and the right inlets (3.0 μL each), while F-SA solution from the center inlets (6.0 μL). As a result, a bright line appeared along the center line of the left microchannel, where the B-tar solution had been flowing. The fluorescence increased with increasing concentration of B-tar. The LOD was estimated to be 2.2 pM, which was better than that of no-LFDA control using the same microfluidic chip, 2.7 nM, by three orders of magnitude.

Next, we tested the discrimination of an SNP in a 56-base sequence (Fig. 6C). After blocking, the target DNA, which represented wild type (WT) or mutant type (MT), was injected from the center inlets (1.0 μL), while biotinylated probes for WT (BP-WT) and MT (BP-MT) were injected from the left and the right inlets, respectively (0.5 μL each). Next, the LFDA was carried out in the same way as the B-tar detection described above. As a result, when the target concentration was higher than 30 pM, fluorescence signal of the matched combination (left channel for WT and right channel for MT) was stronger than the other. The difference became insignificant below 30 pM for MT and 10 pM for WT. These LOD values are worse than that of B-tar, 2.2 pM, probably because of a cross-reaction between the single-base mismatched sequences.

The power-free microfluidic chip was also used for other kinds of DNA analysis. Specifically, methylated DNA, which is important for epigenetics research as well as disease diagnosis, was detected by sandwiching the target DNA between immobilized CP DNA and an anti-methylcytosine antibody. Another series of work is based on non-cross-linking (NCL) aggregation of double-stranded DNA-functionalized gold nanoparticles (AuNPs). This aggregation phenomenon to LFDA, because LFDA is based on aggregation of F-SA and B-anti-SA in the first place. In the case of NCL aggregation, the first layer of AuNPs is probably adsorbed onto the microchannel wall nonspecifically, and then new AuNPs are further deposited from the solution by NCL aggregation. (Historically, this AuNP deposition phenomenon was discovered...
Among them, qRT-PCR is now the standard technique for miRNA profiling, such as quantitative reverse transcription polymerase chain reaction (qRT-PCR), which is extremely sensitive detection, and requires the least equipment means an improvement from the previous work by three orders of magnitude. The total assay time was 20 min.

4-2 MicroRNA

MicroRNAs (miRNAs) are non-protein-coding single-stranded RNAs with typical lengths of ∼22 bases. MiRNAs are intensively studied as new biomarkers for diagnosis of various diseases including cancer, partly because some miRNAs circulate in human body fluids in a very stable manner. Major technical methods for miRNA profiling, such as quantitative reverse transcription polymerase chain reaction (qRT-PCR), deep sequencing, and microarrays, have their own strengths and weaknesses. Among them, qRT-PCR is now the standard technology for diagnostics purposes, because it enables extremely sensitive detection, and requires the least equipment and the shortest time. However, qRT-PCR still takes several hours to complete. Therefore, it is not ideal for POCT.

Toward POCT of miRNAs, the use of the power-free microfluidic chip has been explored. First, a preliminary study was conducted without any amplification step. Because miRNAs are short, their strong binding to probe DNAs cannot be anticipated. In this case, to achieve sensitive and specific detection, probe sequences and experimental protocol must be carefully designed. This preliminary study focused on the order of the hybridization steps to the two probe DNAs: surface-immobilized capture probe (CP) and fluorescently labeled detection probe (DP). Using miR-21 as the target sequence, we studied two protocols. In Protocol 1, the target was hybridized to DP in a test tube, and then the target-DP complex was hybridized to CP in the microchannel. In Protocol 2, the target was first hybridized to CP, and then DP was hybridized to the captured target. As a result, Protocol 1 yielded much stronger fluorescence signal than Protocol 2. The LOD values with Protocol 1 and 2 were 0.62 and 5.3 nM, respectively. This difference was attributed to the coaxial stacking effect between the ends of CP and DP. In general, the coaxial stacking interaction occurs between neighboring terminal bases of two strands that are contiguously hybridized to another strand, and this interaction stabilizes the hybridization between the three strands.

To improve the LOD of miRNA detection on the power-free microfluidic chip, LFDA was adopted. The microchannel design was the same as that for DNA detection (Fig. 6). The CP pattern on the glass surface was modified from the disc shape to a line shape (Fig. 7), which had been formed using another PDMS microchannel. The left half of the double-Y shaped microchannels was used for the miRNA sample, while the right half was used for the blank reference. The experimental protocol is summarized in the table in Fig. 7. First, the blocking buffer was injected from all the inlets. Next, the miRNA sample, the biotinylated DP DNA solution, and the blocking buffer (as the blank reference) were injected from the left, the center, and the right inlets, respectively. At this step, the miRNA sample flow so that the sample contacted all of the CP lines. The sample contained the three miRNAs at varying ratios, while the DP solution contained the three corresponding DP DNAs at a uniform concentration. After LFDA, the fluorescence intensity at each CP line was evaluated. As a result, no cross-reaction between these three miRNAs was observed. This detection method probably has excellent specificity, as long as the target sequences have little similarity. The LOD values were 48 fM, 1.4 pM, and 0.14 pM for miR-16, -21, and -500a, respectively.

The specificity of miRNA detection on the power-free microfluidic chip was further studied under various conditions. First, using a human miR-204 detection system, non-specific responses from mouse miR-211 (1-base mismatch) and human miR-211 (2-base mismatch) were examined. The 2-base mismatch sequence produced no significant signal over all of the tested range (1 pM - 10 nM), while the 1-base mismatch sequence produced certain non-specific signals at very high
concentrations (1–10 nM). In fact, these concentrations were much higher than the LOD of the authentic target (miR-204), 3.4 pM, and therefore this level of non-specific signals are considered to be reasonable and acceptable for many applications. Next, the specificity of mature miRNA detection against its precursor, pre-miRNA, was investigated. In living cells, miRNAs are produced by cleavage from pre-miRNAs, which form hairpin structures with typical lengths of around 70 bases. We tested four microfluidic chips designed for detection of mature miRNAs using excess amounts of corresponding pre-miRNAs. As a result, observed non-specific responses were very low. Interestingly, the non-specific response increased when the hairpin structure of the pre-miRNA was improperly folded. (This was achieved by specialized thermal treatment.) This suggests that the properly folded hairpin structure prevents the pre-miRNA from non-specific binding to CP or DP. Finally, detection of endogenous miRNAs from a complex biological sample was carried out. From human leucocyte total RNA, which was commercially available, miR-16, miR-451, and miR-223 were detected using corresponding microfluidic chips. As a negative control, cel-miR-39, which is found in C. elegans, was chosen. The results were verified with qRT-PCR. As shown in Fig. 8, the two measurement results, by microfluidic chips and by qRT-PCR, agreed very well, especially for highly expressed miRNAs, miR-16 and miR-451. For less expressed miR-223, the agreement was only moderate, and the error bar was rather large. These results indicate that further improvement in sensitivity is necessary for accurate detection of miRNAs with low expression levels. To improve the sensitivity, various efforts have been continued. For example, optimization of the buffer composition led to an improvement of the LOD by 10- to 30-fold.

5 Conclusion and Perspective

The power-free microfluidic pumping technology utilizing degassed PDMS and its application to the analysis of biomarkers, such as proteins and nucleic acids, have been reviewed. This technology enabled the use of microfluidic chips without external pumps and valves, which have been the biggest obstacles for realization of POCT on microfluidic chips. The biomarker analysis on the power-free microfluidic chip was possible with a small sample volume of ~1 μL and a short analysis time of ~20 min. A new signal amplification method specialized for microfluidic chips, LFDA, was very effective to reduce the LOD. In fact, LFDA realized LOD values as low as 0.15 pM for immunoassay of CRP, and 48 fM for surface hybridization assay of miR-16.

For the commercialization of POCT on the power-free microfluidic chip, there remain several technical challenges. Especially for the analysis of miRNAs, LOD should be further reduced, because some miRNAs in body fluids are present in ultra-trace concentrations of around 1 fM. Another technical challenge is miniaturization of the optical setup. The studies described in this article were conducted using a conventional fluorescence microscope for signal readout. For POCT, the microscope is obviously too large and expensive, and has many unnecessary functions. By cutting those functions, a small and inexpensive fluorescence measurement device is currently under development.

6 References

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