Biomarker analysis on microchips

— Development of POCT device for multi-marker analysis —

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Point of care testing (POCT), the analysis of biomarkers at the patient's side, is a continuously expanding trend in the practice of laboratory diagnosis. Although some POCT devices for the analysis of blood glucose and/or several infectious diseases have been developed, many laboratory tests in almost all hospitals are contracted out to clinical laboratory companies. However, outsourcing of biomarker analysis is time-consuming, high in cost, and requires much blood and reagents. Consequently we are constructing a biomarker analysis system on microchips for the POCT device. In this paper, we show the core technology for the analysis of biomarkers on microchips, and describe the problems and its solutions in the application of microchips for POCT device.

Keywords : Laboratory testing, biomarker, POCT, microchip electrophoresis, microchip, multi-marker analysis

1 Introduction

To achieve a high quality life of good health and longevity, it is necessary to prevent various diseases by ultra early detection or predictive diagnosis, particularly of lifestyle-related diseases such as diabetes. To do so, it is necessary to monitor several biomarkers related to various diseases on a daily basis at a personal level, and to establish a network and a diagnostic system of the data obtained. To achieve these goals, we are working to construct a device to measure multiple biomarkers that can be used by patients. Ultimately, it is necessary to establish a technology to detect multiple biomarkers that are present in the humoris such as blood, in the setting of people's daily lives and homes. To realize this, the device must: combine the processes of blood sample collection, pretreatment, separation, reaction, and detection; be compact and easy to use so it can be installed and used at home; and also enable analysis of multiple biomarkers for accurate diagnosis. The biomarker analysis conducted to observe the *in vivo* biological or biochemical changes of protein, glucose, and lipid in the blood is exactly the same as the clinical test conducted when a patient visits a hospital.

Recently, there is an increased demand for "clinical tests at patient's side" or point of care testing (POCT) in the field of clinical tests[1]. In current clinical testing, it is rare that the results be available on the same day as the test. Several days are required before the results become available, and this may prevent swift diagnosis and treatment. Other than the fact that the biomarker measurement itself is time consuming, large and expensive precision measurement device is necessary, and the economic burden on the medical institution is great including the cost of labor for lab technicians. Therefore, many medical institutions outsource the clinical tests to private clinical laboratories. Figure 1 shows the schematic diagram of the clinical test outsourcing based on the brain-to-brain loop model suggested by Lundberg *et al*[2][3]. In contrast, POCT allows the sampling and selection of tests to be done at the patient's side, and the results can be obtained directly from the tests conducted on the spot. This enables definitive diagnosis at the patient's first visit to a medical institution, and this will allow a swift start of the treatment, increased treatment efficiency, reduced load of repeat visits, and reduced medical expenses. This in turn will be beneficial to the patient, the medical institutions, and to society. As a benefit for the clinician, it will enable obtainment of useful, on-the-spot information for determining the treatment method, such as the presence of infections or systemic diseases and their clinical conditions in cases that may require emergency surgical intervention. Currently, POCT devices have been developed and introduced in the clinical practice for conditions that

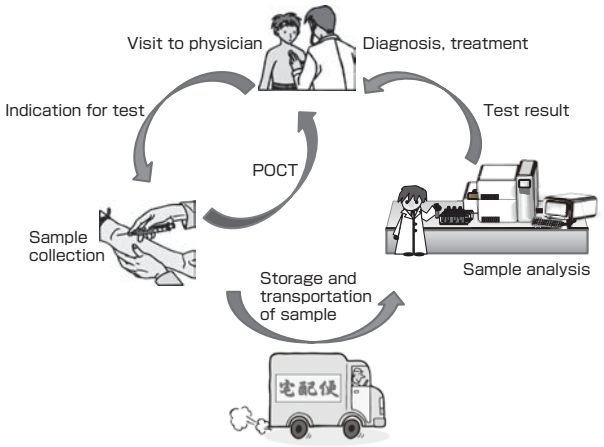

Fig. 1 Flow of tests currently dependent on clinical testing companies.

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require immediate diagnosis, particularly in acute diseases and infections, such as cardiac infarction, influenza diagnostic kit, blood glucose measurement, as well as blood gas measurements in the operating room. However, these products target biomarkers only of specific diseases, and have problems since many detection methods are qualitative. The development a POCT device that can solve these current problems and quantitatively analyze multiple biomarkers will be a core technology for a personal-level multiple biomarker monitoring in the future. Only when the POCT technology is established and its efficacy is recognized in clinical practice, the personal health monitoring technology will be recognized by society (Fig. 2). To do so, it is necessary to realize the POCT device that allows quantitative analysis of multiple biomarkers as soon as possible. Due to the advances in recent analysis technology using nanotechnology, high speed, smaller sample, and higher sensitivity are achieved in testing, as well as the downsizing of device. The development of a device using various microchips is a typical topic of such technological development. Such a device provides benefits to POCT, as explained in the following chapter, and as a personal-level biomarker device. Therefore, as a step for achieving the above goal, we present the application of the nano biodevice to the development of the POCT device by combining the existing microchip core technologies such as the glucose analysis using the commercially-available microchip electrophoresis device for nucleic acid analysis, and the construction of the antigen-antibody reaction system on the micro flow channel using microfluidics. The issues that are yet to be solved will be described from the standpoint of a biological user with clinical experiences.

2 Requirements of the POCT device

The POCT device must allow measurement of a biomarker quickly in the doctor's office or the hospital ward, provide analysis within 30 min[1], possess sensitivity and reproducibility usable for clinical diagnosis, enable measurement equivalent or superior to the current clinical test method, have compactness that enables installation in the office, and be operable for physicians while interviewing patients. Regular blood tests require several ml of blood per test item, and that is not only stressful to the patients, but also is costly since it necessitates large quantities of reagents for the test tube analysis. Therefore, analysis with microscale samples is in demand not only in POCT but also in clinical tests in general. Also, since the test samples include blood, the device material must be easily sterilized after testing. Also, considering the needs of a device that allows the detection of multiple test items, we focused on the microchemical chip technology based on the microfabrication technology, or the microchemical analysis system where the procedures for chemical and biochemical analyses such as the pretreatment, separation, reaction, and detection are integrated on a microchip of few centimeter square size. Aiming at the application to the POCT device, we clarified the conditions for optimal detection of commercially-available individual biomarkers using the microchip electrophoresis and microfluidics, and worked on the on-chip mounting of the biomarker detection system.

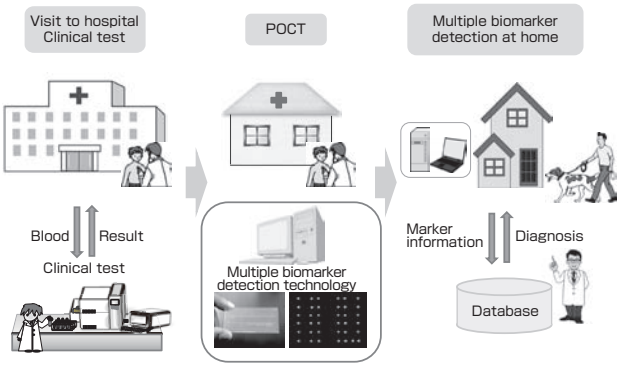

Fig. 2 Requirements for POCT and the schematic diagram of the integrated microchip substrate.
The plasma separation mechanism is installed onto the microchip substrate. There are flow channels for microchip electrophoresis and microfluidics system.

3 Application of the microchip substrate to the POCT device

3.1 Construction of the biomarker measurement method using the glucose analysis by microchip electrophoresis

3.1.1 Application of the microchip electrophoresis to biological and biochemical analysis

Microchip electrophoresis devices have been developed and are commercially available. In these devices, electrophoresis is conducted in the micro flow channel with μm-level width and depth formed on the microchip made of plastic or glass material of a few centimeter square size using the microfabrication technology for semiconductors. Compared to the conventional electrophoresis method for the separation analysis of nucleic acid and proteins, the microchip electrophoresis has high separation capacity by application of high voltage, because the sample volume can be reduced by using the micro flow channel, and because the efficiency of heat release during electrophoresis is increased due to the increased surface volume against the sample volume in the flow channel. Moreover, higher sensitivity can be achieved by using the LED-excited fluorescence detection system. These devices, however, are not sufficiently diffused in the biology and biochemistry labs of the universities that are expected to be the main users. The main reasons are because their usage is limited to the separation analysis of nucleic acids, the price of the electrophoresis device is higher compared to the conventional agarose electrophoresis, and the cost of microchips and gel necessary for the analysis per sample is about 200 times higher. Therefore, we attempted using the device for purposes other than nucleic acid separation without changing the electrophoresis chip, the device, or

the analysis software, by optimizing the electrophoresis conditions by considering the gel and buffer solution compositions. We found that in the various experimental methods conducted daily in the biology and biochemistry laboratories, the presence of the proteins such as enzymes and ions such as Mg^{2+} ions necessary for restriction enzymatic activities did not affect the electrophoresis, by conducting the electrophoresis analysis after the on-chip restriction enzymatic treatment using the sample reservoir as a reaction field. Based on this result, we designed the on-chip restriction enzyme treatment method, and conducted quick restriction fragment length polymorphism (RFLP) analysis. We also reported that a biology researcher could conduct the mitochondrial membrane potential measurement, as well as application to synthetic RNA analysis and DNA ligation reaction analysis, simply by changing the electrophoresis condition, without changing the electrophoresis device or the analysis software. We also reported the high applicability of the microchip electrophoresis to biological and biochemical analyses utilizing the advantages, as it allows not only for nucleic acid separation analysis but also rapid, small sample, and high sensitivity analysis and various enzyme treatments[4]-[8]. These results indicate that the microchip electrophoresis can be applied to various experimental procedures, and cost reduction can be expected.

3.1.2 Application of the microchip electrophoresis to blood glucose analysis

Based on these findings and considering the application to POCT, we applied the blood biomarker analysis using the commercially-available microchip electrophoresis device and microchip. The Hitachi SV1100 was used as the microchip electrophoresis device, because it allowed easy handling of the solution by Pipetman due to its 10 µl reservoir capacity, and the gel and buffer solution could be changed easily. The supplementary chip was used as the microchip. Figure 3 shows the *i*-chip made of polymethylmethacrylate (PMMA) to be used with the SV1100. The *i*-chip has three micro flow channels with width of 100 µm and depth of 30 µm, and simultaneous analysis of three samples was possible (Fig. 3A).

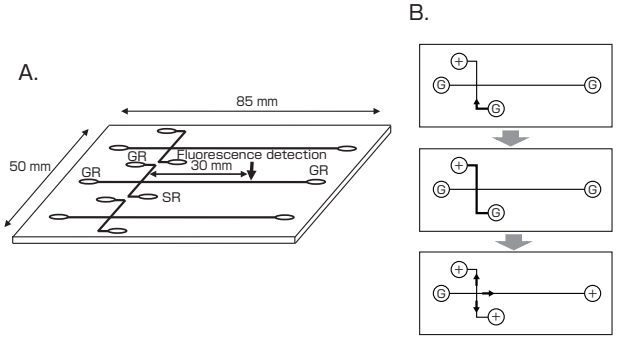

Fig. 3 Schematic diagram of Hitachi *i*-chip (A) and sample separation in the cross flow channel (B).
The "+" indicates the anode, "G" indicates the ground, and arrows show the migration direction of the sample DNA.

The electrophoresis procedure is simple. After adding the gel from the gel reservoir (GR), total 10 µl sample solutions including the internal control DNA were placed in the sample reservoir (SR). Electrophoresis and separation were conducted, and the separation and analysis of the DNA were done by fluorescence detection (Fig. 3B). In this microchip electrophoresis, the detection sensitivity was about 10 times higher than that of conventional agarose gel electrophoresis with fewer samples. The analysis result was obtained in a few minutes after the start of the electrophoresis, and the DNA could be separated with error of only a few bases. We focused on the high DNA separation capacity including the glucose structure of the microchip electrophoresis, and used the supplied DNA analysis software for the analyses for blood glucose and amylase that have glucose structure or use glucose as enzyme substrates[9][10].

In the blood glucose measurement, we reported that the blood glucose could be specifically detected, after directly fluorescence-labeling the glucose by adding the fluorescent pigment 2-aminoacridone (AMAC) to the blood plasma, and then charging the glucose negatively using the boric acid buffer solution as the driving force of electrophoresis (Fig. 4A)[8]. It was found that the separation and analysis by electrophoresis of fluorescence-labeled glucose could be done in the blood plasma sample in which diverse proteins and other substances were present. This method had detection limit of 0.92 µM, allowed quantitative detection in the range of 1~300 µM, and enabled detection of blood glucose as accurate as the blood glucose level obtained by the conventional clinical test. Moreover, it showed high reproducibility both in within-a-day and between-days reproducibility, and indicated the possibility of practical application to blood glucose measurement by microchip electrophoresis. In the hexokinase-G-6-P-dehydrogenase method used in current clinical tests, there is a major problem where the value higher than the actual glucose measurement is obtained due to the presence of the disaccharide maltose in the infusion. However, by using the microchip electrophoresis, the monosaccharide glucose and the disaccharide maltose can be easily identified due to the difference in migration time[11]. As a result, the risk of hypoglycemia due to the false high-value reading of the glucose measurements in patients receiving infusion containing maltose can be prevented.

3.1.3 Measurement of blood amylase activity by microchip electrophoresis

Blood amylase is a biomarker used in the diagnosis of pancreatitis and sialadenitis. Amylase hydrolyses the glycoside bond and converts starch into glucose, maltose, and oligosaccharides. In the current clinical test, the oligosaccharide is used as the enzyme substrate and a qualitative measurement is done by the colimetric method[12]. Since it is already known that amylase is hydrolyzed

into maltohexaose (G6) and maltotriose (G3) that are oligosaccharides, we focused on the high separation capacity of the fluorescence-labeled glucose using the microchip electrophoresis that became apparent in the blood glucose measurement. We used the APTS-G6 that was fluorescence-labeled by 8-aminopyrene-1,3,6-trisulfonic acid (APTS) as a substrate, separated the breakdown product APTS-G3 by microchip electrophoresis, and then quantitatively measured the amylase activity (Fig. 4B)[10]. Here, the boric acid buffer solution was used as the driving force of migration, as in the blood glucose separation. In this method, the quantitative detection of blood amylase activity was possible in the range of 5~500 U/L at detection limit of 4.38 U/L. There are two isozymes of blood amylase originating from the pancreas or the salivary gland. By conducting the plasma pretreatment using the anti-amylase antibody of salivary gland origin for the differential diagnosis of pancreatic disease, the specific measurement of pancreatic-origin amylase activity became possible. When the plasma sample was used, it was shown that the amylase activity measurement was accurate as the current clinical testing method, and this indicated the possibility for practical application of the microchip electrophoresis in amylase activity measurement.

In the glucose and amylase measurements described above, about one hour is necessary for blood glucose labeling and enzyme treatment of APTS-G6, and the treatment time must be reduced for application as POCT. Therefore, it is necessary to shorten the detection time by achieving high sensitivity by changing the fluorescence material or the detection system. However, the blood glucose and amylase activity are measured using the supplemented DNA analysis software, after the fluorescence-labeling and electrophoresis of the plasma using the commercially-available microchip electrophoresis device and the supplemented electrophoresis chip, or by mixing the fluorescence-labeled oligosaccharide with the plasma and then conducting electrophoresis. The major advantages are that the quantitative detection can be done extremely easily, and that it has accuracy and reproducibility equivalent to the current clinical test method. Also, only μl level of plasma sample is required, the device is compact, and the plastic substrate can be sterilized in an autoclave. Combining all these factors, the high potential of the microchip electrophoresis in biomarker analysis such as blood glucose and amylase is indicated. However, the glucose and amylase measurements are relatively inexpensive at 110 yen per test as covered by the National Health Insurance system, and the economic feasibility is low when only one item is tested with the microchip electrophoresis. However, sufficient economic feasibility can be maintained by measuring a combination of multiple test items, such as the detection of various blood proteins that will be mentioned later, on one chip for a particular disease.

3.2 Construction of the antigen-antibody reaction system on the microchip substrate

3.2.1 Construction of the sandwich ELISA on the micro flow channel

Many of the biomarkers in the blood are various metabolites and proteins, and specific detection is possible even in blood in which many foreign substances are present. In the current clinical test methods, the antigen-antibody reaction system that does not require molecule sorting by electrophoresis is widely used. In the current clinical test methods, the antigen-antibody reaction using 96-well plate is generally used, but over one hour is needed for the reaction time, and several tens of μl of samples are necessary. Therefore, we attempted construction of the antigen-antibody reaction system in the

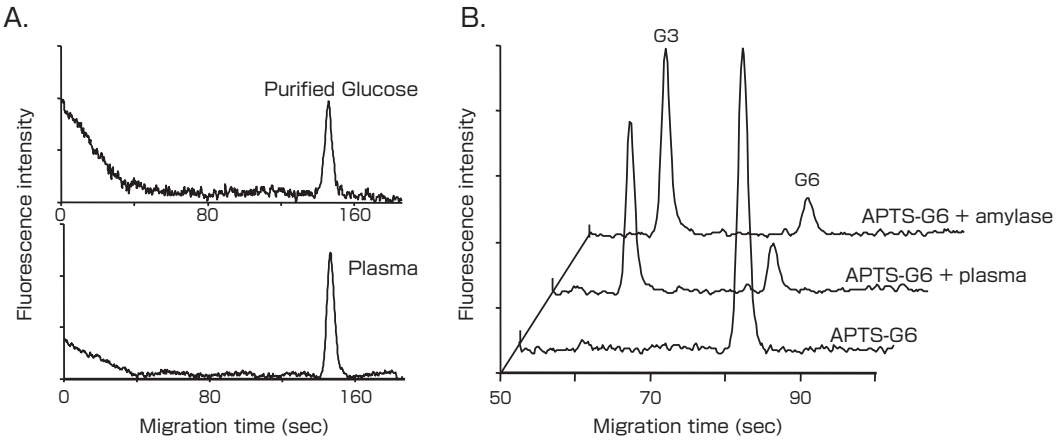

Fig. 4 Measurements of blood glucose (A) and amylase activity (B) using the microchip electrophoresis.

(A) A peak for plasma glucose was observed in a similar migration time as the glucose preparation. The blood glucose concentration was measured by calculating the calibration curve from the fluorescence intensity of the glucose preparation with known concentration.

(B) A single peak was observed in the solo electrophoresis of APTS-G6. Treatment of APTS-G6 with purified amylase, the single peaks of APTS-G6 and APTS-G3, its breakdown product, were observed. By reacting the APTS-G6 with plasma, it was broken down into APTS-G3 by the action of blood amylase. The blood amylase activity was measured by calculating the calibration curve from the fluorescence intensity corresponding to APTS-G3 by treatment of APTS-G6 with amylase with known concentration.

micro flow channel on the microchip to reduce the sample quantity and to reduce the antigen-antibody reaction time by molecular scattering using the microspace. We investigated the sandwich enzyme-linked immuno-sorbent assay (ELISA) method (Fig. 5A) that is widely used for biomarker detection and has excellent quantitative quality.

The blood procollagen I carboxyterminal propeptide (PICP), which is a biomarker for osteoporosis and cancer metastasis in which highly specific antibodies are commercially available, was selected as the measurement model. In the sandwich ELISA method, the primary antibody is fixed on to the solid phase[13]. In the conventional test, the 96-well plate (Fig. 5B) was mainly used as the solid phase, but here, the microchip (Fig. 5C) was used. After fixing the antibody and conducting blocking[Term 1], the plasma sample or the purified PICIP with known concentration and the peroxidase-labeled secondary antibodies were added. Fixing on to the solid phase was done via the bonding of PICP bonded to the labeled secondary antigen to the PICIP primary antibody. After washing away the labeled secondary antibody that did not bond to the antigen, peroxidase substrate was added, and the chemiluminescence was detected by a CCD camera. When using this as the POCT device, the user carries out the procedure after the blocking procedure. In the conventional 96-well plate, three hours was needed for the antigen-antibody reaction using 20 µl plasma. The cyclic olefin copolymer (COC) (Sumitomo Bakelite Co., Ltd.) substrate, in which the surface is treated to fix the protein and has three micro flow channels on one microchip, was used as the microchip substrate. The introduction of each solution at µl level into the micro flow channel was done using the Pipetman. Blocking was done after introducing the primary antibody from the sample well (1) in the direction of (2) and fixing it, and the antigen and the peroxidase-labeled secondary antibody were introduced in the direction from (3) to (2). Washing was done after the antigen-antibody reaction, the enzyme substrate was added in the direction from (1) to (2), and chemical luminescence was detected. The amount of plasma necessary per micro flow channel was 1 µl or less, and the antigen-antibody reaction time was 30 min. Dramatic reduction of the detection time and the sample volume was realized compared to the conventional method.

For the antigen-antibody reaction in microspace, the method in which the micro-beads with diameter of several µm were fixed on the antibody, and then introducing and fixing the beads in the micro flow channel had been reported. In the beads method, there are issues such as a necessity of designing a micro flow channel with a complex shape to contain the beads in the channel. Therefore, we selected the method of fixing the antibody directly to the micro flow channel surface. As shown in Fig. 5C, the increased chemiluminescence intensity was observed in accordance to the concentration of the purified PICP, but the uneven luminescence in the same flow channel was observed, and it could be seen that quantitative property was not maintained. The reasons were thought to be: 1) uneven fixing of the primary antibody in the flow channel surface, and 2) insufficient washing or partial residue of labeled secondary antibody in each steps of blocking. In the Y-shaped flow channel used (Fig. 5C), the increased chemical luminescence was observed particularly at the fork point of the flow channel. It was necessary to design a flow channel that could be washed easily. Therefore, we attempted improvement of the quantitative property by fixing the primary antibody to the specific part of the micro flow channel using the inkjet for 1), and by increasing the washing efficiency by changing the flow channel design for 2).

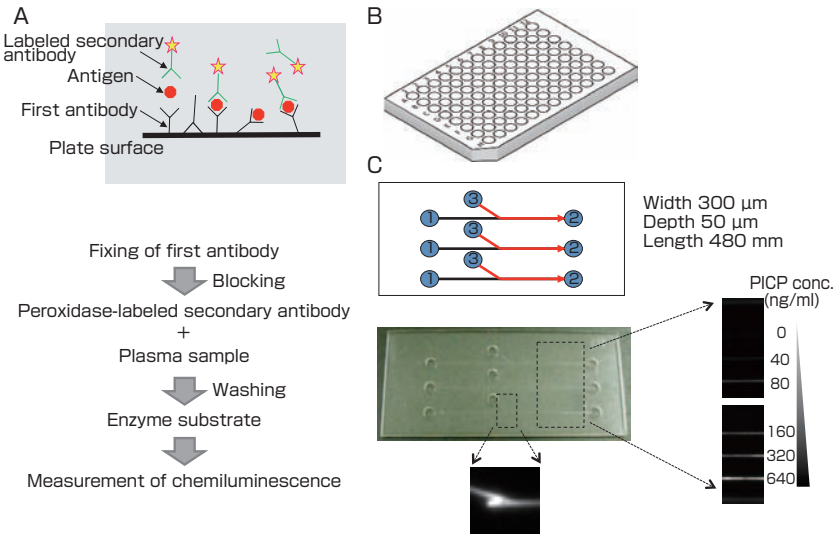

Fig. 5 Antigen-antibody reaction by sandwich ELISA method.
Principle of the sandwich ELISA method and its experimental procedure (A), 96-well plate (B), and PICP detection in the micro flow channel (C).

3.2.2 Fixing the antigen to the micro flow channel surface using the miniaturized inkjet

To fix a certain amount of antigen on to an arbitrary part on the micro flow channel, miniaturized inkjet that can be programmed to discharge ultralow volume of solution at pl level was used. The miniaturized inkjet used was the piezo-driven Pulse Injector (Cluster Technology Co., Ltd.) (Fig. 6). This inkjet device could discharge 65 pl of diluted anti-PICIP primary antigen in one droplet. When 100 droplets of primary antibody was discharged and fixed using this device, the droplet diameter reached approximately the width of the flow channel, and the anti-PICIP primary antibody was fixed (Fig. 6). As mentioned earlier, in the design of the micro flow channel with a fork, nonspecific chemiluminescence[Term 2] was seen, where a strong chemiluminescence was observed in the fork part due to the problem of washing, and it was difficult to maintain the quantitative property. Therefore, four linear micro flow channels that allowed easy washing was formed on one COC microchip (Fig. 7A) to construct the quantitative detection system. After discharging and fixing the primary antigen to the micro flow channel surface using the inkjet, blocking and washing were done in the direction of (1) to (2) to prevent the nonspecific adsorption and residue antibody, and after 30 min of antigen-antibody reaction, the chemiluminescence was detected using a CCD camera (Fig. 7B). In this reaction system, the amount of plasma needed per micro flow channel was 1.8 µl and the antigen-antibody reaction time was 30 min. These were 1/10 or less and 1/6, respectively, compared to the conventional 96-well plate method, and a low sample, high-sensitivity detection system was constructed. As a negative control, the antibody for the cardiac infarction marker, heart type fatty acid binding protein (H-FABP), that did not recognize the PICIP was discharged and fixed on to the micro flow channel surface using the inkjet. No nonspecific luminescence was observed for H-FABP, and good quantitative property was observed in the concentration range of 0~600 ng/ml (Fig. 7B, C). In the case where the plasma sample was used, rapid, low-volume, and accurate detection system that allowed measurement accuracy equivalent to the current sandwich ELISA method using the 96-well plate was possible. By conducting the antigen-antibody reaction in the micro flow channel, we were able to construct a blood protein detection method that could be applied to the POCT technology.

In the method where the antibody is discharged and fixed in the micro flow channel using the miniaturized inkjet, the discharge and fixing of an arbitrary amount of antibody on to an arbitrary part becomes possible (Fig. 7D). The principle of the blood protein detection by sandwich ELISA method is basically similar regardless of the type of biomarker. Multiple types of antibody solution can be discharged by changing the head of the inkjet including the antibody solution, and multiple biomarker detection is possible from only 1.8 µl plasma sample in one micro flow channel. Currently, we are investigating the concentrations of the various primary and secondary antibodies as optimal conditions of the antigen-antibody reaction system to quantitatively detect multiple types of blood biomarkers on one micro flow channel. We are aiming to fabricate the multi-marker detection microchip, particularly the diagnostic chip for diabetes and osteoporosis that are gathering attention as life-style related diseases. For the diabetes diagnosis, in addition to blood glucose, measurements of insulin and high-sensitivity CRP that could be detected by antigen-antibody reaction could be mounted on the chip for accurate diagnosis with very small amount of blood. In osteoporosis, the detailed state of the disease can be observed by measuring both the osteogenesis marker PICP and the absorption marker NTx. Considering the cost advantages, the current insulin test is

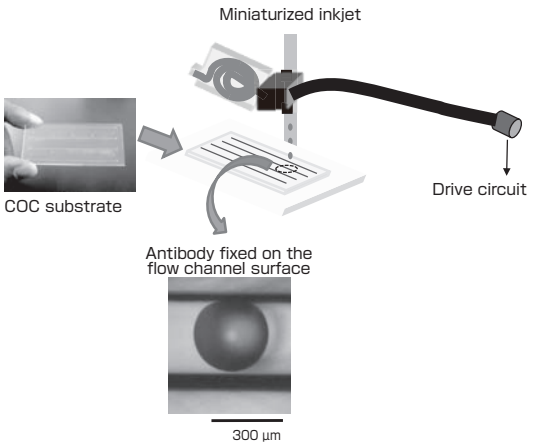

Fig. 6 Schematic diagram of the antibody fixing on the micro flow channel using the miniaturized inkjet, and the antibody fixed on the flow channel surface.

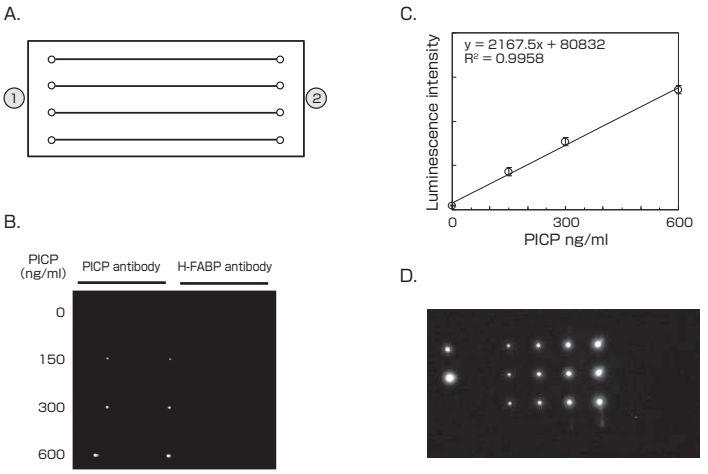

Fig. 7 Schematic diagram of the microchip substrate with micro flow channel (A) and the PICP detection image using this chip (B), calibration curve (C) and the image of chemiluminescence when antibody of arbitrary amount is fixed on each flow channel (D).

2640 yen while CRP measurement is 1560 yen, and adding the blood glucose measurement, the total is 4310 yen for the three items. In osteoporosis, the PICP measurement is 1700 yen and the NTx measurement is 2900, requiring a total of 4600 yen. The cost of the antigen-antibody reaction system is dominated by the cost of antibody used as the reagent. Therefore, if the antibody fixing method using the inkjet is employed, the amount of antibody used is about 1/10,000 in PICP compared to the 96-well plate method, and the cost of antibody can be reduced drastically. Also, by fixing multiple types of antibody on to one micro flow channel, the amount of detection reagent used can be greatly saved, and used with plastic substrate made of inexpensive material, sufficient economic feasibility can be expected.

4 Future issues

We have been working to construct the biomarker detection system based on the existing technologies such as the microchip and the electrophoresis using microchip from the standpoint of users in the biology field. For the microchip electrophoresis, we were able to present the high potential of the current individual technologies in relatively short time, without spending time and effort on the development of the new device and software, but using the commercially-available chip, electrophoresis device, and analysis software for application to the biology and biochemistry experiments and clinical tests. However, for the realization of the POCT device, besides the biological approach, it is necessary to collaborate with engineers and researchers across the fields, including engineering and medical fields focusing on microfabrication, information fields for future database construction.

While we were able to construct the core technology for the biomarker analysis on the microchip for glucose and protein, the following issues remain before the POCT device can be used in actual clinical practice. In the above microchip, the analysis is conducted after the conventional blood cell separation by centrifugation, and then adding the plasma components to the microchip. Therefore, to do the blood test during interviews by physicians in clinical practice, the blood cell separation process must be simplified. Therefore, it is required that the analysis, including blood cell separation, can be accomplished simply by adding the whole blood to the microchip. To enable tests with ultralow volume blood at μl level, we are working on an on-chip plasma separation system with a micro flow channel that incorporates a filter for separating the erythrocyte components from the blood drawn using currently available disposable microsampling needles. Moreover, we must construct a solution sending system using a micropump to send the necessary amount of plasma automatically and quantitatively to the sample well in the flow channel of microchip electrophoresis or microfluidics. Also, for the realization of the multiple biomarker detection chip, a complex micro flow channel design is required. In the design, it is necessary to install the separation and analysis systems that have different principles on one microchip, including the electrophoresis system where the substance is separated by the difference of migration speed due to the charge, size, and shape of the substance, and the micropump[Term 3] used in antigen-antibody reaction system by sending ultralow volume liquid. For this, it is necessary to collaborate with companies with expertise in microfabrication technology including plastic forming.

In addition to the above technological issues, a prototype of the POCT device will be fabricated as soon as possible, by integrating and constructing the peripheral technology such as the development of the detection system and analysis software. In this case, the subject will be the diagnostic chip for diabetes and osteoporosis that affect several million to ten million patients among Japanese adults. The efficacy as a POCT device is verified by comparisons with current clinical test data through joint research with university hospitals and specialized hospitals. Data will be collected to obtain approval of the Ministry and Health, Labour and Welfare based on the Pharmaceutical Affairs Law as a medical test device. After introducing this POCT device to clinical practice, we shall work on the introduction of the biomarker measurement device to monitor health at home.

Terminology

Term 1. Blocking: Prevention of the nonspecific bonding of the antibody to proteins other than the antigen proteins or to solid phase surface. Bovine serum albumin, gelatin, and skim milk are often used as the blocking agents.

Term 2. Nonspecific luminescence: Luminescence caused by the breakdown of peroxidase enzyme substrate, after the peroxidase-labeled secondary antibody bonds nonspecifically to the protein or solid phase surface, due to insufficient blocking or cleansing. It becomes background noise.

Term 3. Micropump: Liquid control element that generates pressure to move the ultralow volume of liquid.

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Completed the course at the Graduate School of Dentistry, The University of Tokushima in 1990. Doctor of Dentistry. Worked as a dentist specializing in periodontal disease at the Faculty of Dentistry, The University of Tokushima from 1990 to 2002, and studied the genetic analysis of the periodontal pathogenic factor. Engaged in research for the biological analysis using the microchip electrophoresis as an associate professor of genetic expression field at the Institute for Genome Research, The University of Tokushima until 2004. Joined the Health Technology Research Center, AIST Shikoku as a senior researcher in 2006. Leader of Biomarker Analysis Team from 2007. Leader of Biomarker Analysis Group, Health Research Institute, AIST from 2010. Works on application of the microchip to biomarker analysis including electrophoresis. In this paper, constructed the biological analysis method using the microchip and coordinated the overall concept.

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Joined the Shikoku National Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry in 1988. Engaged in research for the mechanical system control and the measurement technology using ultrasound. Became a senior researcher of AIST in 2001. Doctor (Engineering) from The University of Tokushima in 2002. Senior researcher of the Biodevice Team, Health Technology Research Center, AIST Shikoku from 2005. Senior researcher in Health Research Institute, AIST from 2010. Currently studies the microfabrication technology and the ultralow-volume droplet control technology for biodevice construction. In this paper, worked on the discharge and fixing of antibody using the miniaturized inkjet.

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Leader of Biodevice Group, Health Research Institute, AIST from 2010. Specialty is microfabrication using lasers. Develops the plastic disposable chip, the units to be embedded in integrated chip, and others to enable multi-item diagnosis using blood, to realize a device that can numerically express the human health condition. In this paper, constructed the discharge and fixing of antibody using the miniaturized inkjet for multi-marker analysis.

Discussions with Reviewers

1 Practical use

Question (Kazunori Nakamura, Evaluation Department, AIST)

The authors mention, "There are many issues that remain before this can be actually used as a POCT device in clinical practice," and I think the technology is still quite far from practical use. You should add this point to the title and the introduction of the paper to let the readers know.

Please provide an analysis of the many remaining issues, organize their solutions and the actual efforts, and summarize the future direction. Also, before the device and the method can be used in actual clinical tests, there are issues of cost and approval by the Ministry of Health, Labour and Welfare, as well as its quantitative property and reliability. Since getting the MHLW approval involves setting the National Health Insurance points, it is important to establish the cost efficiency compared to the existing method. What is your thought on this?

Answer (Masatoshi Kataoka)

The future issues were described in chapter 4. The technologies include (1) mounting the blood cell separation system on to the chip, (2) construction of pump system using the micropump, and (3) micro flow channel design. The prototype of the device will be fabricated, and its efficacy as a clinical test device must be demonstrated. Also, we described the necessity of the approval as a medical test device according to the Pharmaceutical Affairs Law. On the issue of cost, we explained that there is sufficient economic feasibility when compared to the current test costs in terms of the NHI points.

For the title, we changed it to emphasize the development of the core technology.

2 Comparison with current technology

Comment (Kazunori Nakamura)

This technology is applied to the measurement of blood glucose and amylase activity. Particularly for blood glucose, the glucose sensors that the patient can use daily are already widely in use. Therefore, please state the issues in the currently used methods, and clarify the things you did to solve those problems. You also mention that the method in this study has equivalent performance to the current clinical test method, and therefore, I think you should explain the future developments such as whether this method will replace the current clinical test, whether there is possibility of diffusion as a POCT device, and the path to practical use including the cost aspects.

Answer (Masatoshi Kataoka)

As you mentioned, there are blood glucose sensors that are commercially available as POCT device. However, in the glucose measurement using the hexokinase-G-6-P-dehydrogenase method, higher-than-actual glucose value may be indicated because the disaccharide maltose is identified as monosaccharide glucose. This is a major issue when the patient is receiving infusion that contains maltose (there are actual fatal cases due to hypoglycemia). In such cases, the identification of monosaccharide and disaccharide by electrophoresis based on the migration time provides great clinical advantage.

For amylase measurement, we described that the conditions needed as a POCT device are cleared, such as quantitative property, simple operation, smaller amount of samples, compactness of the device, and chip that can be autoclaved. In terms of cost, we described that the cost of the electrophoresis device will be reduced due to the high universal application of the microchip electrophoresis to various experimental procedures. Also, we explained that in clinical tests, because the costs of blood glucose and amylase measurements are already low in the NHI, the cost of a new device will not be feasible for single tests, but the cost competitiveness will be sufficient if it is used as a multiple analysis chip where several test items are combined.

3 Performance of individual technologies

Comment (Kazunori Nakamura)

You indicated the problem of sandwich ELISA currently used. However, it is not really clear how effective the reduction of measurement time is in the diagnosis of the long-term disease like osteoporosis. You write that the reaction time was reduced from conventional three hours to 30 minutes, but the measurement principles are the same as the antigen-antibody reaction and enzyme reaction, and you don't sufficiently explain why it is reduced even if they are basically the same principle.

Comment (Motoyuki Akamatsu, Human Technology Research Institute, AIST)

Please explain, why the microchip electrophoresis can use small amounts of samples and have high detection sensitivity compared to the conventional agarose electrophoresis.

Answer (Masatoshi Kataoka)

When constructing the antigen-antibody reaction system, the antigen identification capacity of the antibody becomes the issue. We selected the PICP because: it is a marker for osteoporosis, which is a social issue being a life-style related disease with high number of patients (fracture due to osteoporosis may cause geriatric patients to become bedridden); the highly specific antibody is readily available (the antibody is commercially available independently); and the PICP blood concentration is measurable in healthy individuals (data is always obtainable from the blood sample; markers such as inflammatory cytokine will fall below measurement limit in healthy individuals, and data analysis is difficult → difficult to establish an experiment system). We added these points to the paper.

Since the principle of antigen-antibody reaction is basically the same regardless of the marker type, the basics are the same whether it is PICP or any other marker under investigation. Therefore, we selected PICP for investigation because it is an osteoporosis marker for which highly specific antibody is commercially available. The reduction of time from three hours to 30 minutes will allow use in a doctor's office, which is a requirement of POCT. In antigen-antibody reaction, specific bonding starts with the collision of the antibody and antigen in space. In the microspace, the dispersal time is reduced due to the molecular dispersal effect, and this, as a result, is thought to lead to the reduction of antigen-antibody reaction time. We also added this explanation.

For electrophoresis, we stated: "Compared to the conventional electrophoresis method for the separation analysis of nucleic acid and proteins, the microchip electrophoresis has high separation capacity by application of high voltage, because the sample volume can be reduced by using the micro flow channel, and because the efficiency of heat release during electrophoresis is increased due to the increased surface volume against the sample volume in the flow channel. Moreover, higher sensitivity can be achieved by using the LED-excited fluorescence detection system."

4 Approach based on clinical experience

Comment (Motoyuki Akamatsu)

While I understand that the approach from the standpoint of biological researchers with clinical experience is highly valuable, what exactly is based on clinical experience is not explained. For example, one could readily see, even without clinical experience, that the treatment time and device size are issues to be solved. I think you should clearly describe the points seen from the clinical side.

Answer (Masatoshi Kataoka)

In the construction of the personal-level health monitoring system for home use, we are considering an approach for the development of the POCT device, toward the construction of the blood biomarker device that can be used in daily living. Therefore, we are aiming to realize the device as soon as possible, using the microchip substrate that is a currently available technology. We also added that from a clinical standpoint, in cases where emergency surgical procedure is necessary, the device will allow obtaining information useful to determine the treatments, such as the presence and status of the infections or systemic disease.

5 Approach of combining the existing technologies

Comment (Motoyuki Akamatsu)

I understand that the individual technologies are not original, but the combination of these technologies makes this research original. However, when you claim the originality of the combination technology, I would like to see the discussion of why you did not select the other elemental technologies. Overall, there are explanations about the things that were done using the technologies that you selected. However, as a *Synthesiology* paper, I would like you to explain how you selected the technologies.

Answer (Masatoshi Kataoka)

As you indicated, cost reduction can be expected because the commercially-available microchip electrophoresis device, supplementary chip, and analysis software can be used directly for the various biological experimental methods. We explained this point. For the selection of the elemental technologies, we addressed the combination of existing technologies in the selection of the microchip. Also, we explained that the reason for selecting Hitachi SV1100 was because the migration gels and buffer solutions could be changed easily. The reason for focusing on the hydrolysis of G6 and G3 in the amylase measurement was because they could be easily separated by electrophoresis. The reasons for selecting the PICP were because of its high disease specificity (as osteoporosis and cancer metastasis markers) and because highly specific (good) antibodies are commercially available. The antibody specificity is extremely important, and we placed weight on this point. Also, for the antibody fixing method in the antigen-antibody reaction in microspace such as the micro flow channel, we added the comparison with the beads method.

We also described that we were able to demonstrate the potential of the current individual technologies without spending much time, and yet for the future product realization as a device, collaboration with engineering and medical fields is necessary.

6 Amylase of pancreatic and salivary gland origin

Question (Motoyuki Akamatsu)

In section 3.1.2, you explain the necessity of separating the amylase of pancreatic origin and the one of salivary gland origin. However, in actual clinical practice, the presenting symptoms are completely different (swelling occurs in different places) in pancreatitis and sialadenitis, and there is very low chance of missing the diagnosis. Even so, is it necessary to separate the two? Of course, there is the advantage of detecting the disease before the symptoms manifest, but looking at the patients, I think there are very few people with which the disease must be caught before the inflammations occur. What do you think?

Answer (Masatoshi Kataoka)

As you mentioned, the parts affected by pancreatitis and sialadenitis are completely different. Therefore, differential diagnosis can be made easily from the clinical symptoms such as swelling and inflammation, but blood amylase is measured as a marker to see the clinical state of the diseases. As described in the text, there are two types of blood amylase, one of pancreatic origin and other of salivary gland origin. About 40 % originates from the pancreas, and 60 % from the salivary gland. This ratio depends on the individual, age, and gender. Therefore, to understand the clinical state and to determine the treatment efficacy, it is necessary to accurately measure the amylases originating from each organ. The pancreatic-origin amylase is also used as a marker in the follow-up observation of acute pancreatitis, chronic pancreatitis, and pancreatic carcinoma.