RAPID URINE-BASED CLINICAL DIAGNOSIS OF DIABETIC NEPHROPATHY WITH FEMTO-MOLAR SENSITIVITY BY IMMUNO-PILLAR DEVICES

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ABSTRACT

We have developed immuno-pillar devices for rapid and easy-to-use immunoassay, but to improve the speed of assay (5-10 min) and the detection sensitivity (nM-pM level) has still remained as major problems toward the clinical applications of immuno-pillar devices. We report here the second-generation immuno-pillar devices with faster assay within 2 min and pM-fM detection sensitivity. New devices enable us to apply them to the clinical trials for the detection of multiple biomarkers (monocyte chemotactic protein 1 (MCP-1), angiotensinogen (AGT), liver-type fatty acid binding protein (L-FABP)) of diabetic nephropathy in human urine without any pretreatments.

KEYWORDS

Immunoassay, Biomarker, Clinical Samples, Urine

INTRODUCTION

The application of the microchip technologies to clinical diagnosis has many advantages in reductions of the amount of samples, total assay time, costs and so on. This allows us frequent check-ups of various diseases, resulting in the early diagnosis. Typically, the therapy of the patients with late stage takes much cost, extremely long time and physical and mental strain of the patients. For example, late stage diabetic nephropathy is a leading cause of the artificial dialysis. Since the patients with late stage are forced to receive the artificial dialysis three times a week in a hospital, the establishment of an early stage diagnosis method is strongly required. To achieve this, we developed an immunoassay device [1], in which PEG-based hydrogel pillars with antibody immobilized 300000 microbeads were fabricated in a microchannel. Based on the microbeads, we performed the sandwich immunoassay against the markers of diabetic nephropathy. The immunoassay within 2 min and pM-fM detection sensitivity was achieved and our estimations of the markers' concentrations had good correlations with those obtained by the conventional microtiter plate method.

EXPERIMENT

DEVICE

A photograph of the substrate and schematic of the PEG-based pillars are shown in Fig. 1. We call this device "immuno-pillar device". Immuno-pillar structures are fabricated by photo-polymerization of PEG-based polymer with antibody-immobilized microbeads inside a microchannel. In the first-generation immuno-pillar devices [1], we controlled the mesh size (ca. 200 nm) of PEG-based polymer to contain 30,000 microbeads inside a single immuno-pillar, but it leads to slower migration of high-molecular weight biomarkers and lower detection sensitivity. In order to achieve faster assay and higher sensitivity, we controlled the mesh size from 500 nm to 700 nm to contain much more number of beads and accelerate the diffusion rate of biomarkers. As a result, over 300,000 antibody-immobilized polystyrene microbeads are effectively captured inside a single PEG-based hydrogel pillar.

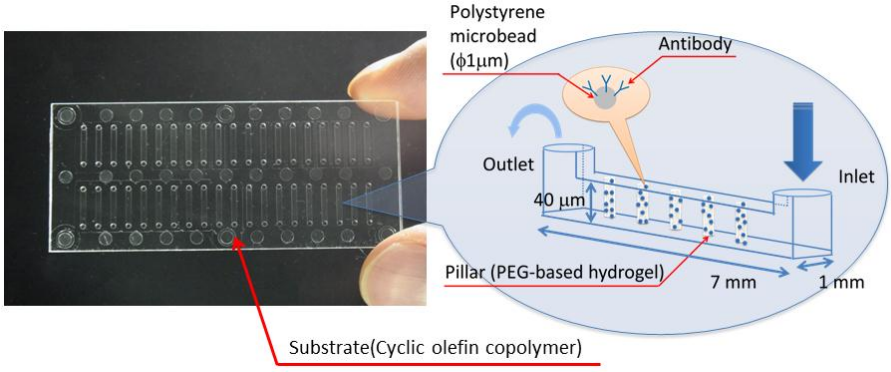

Figure 1: Photograph and schematic of the immunoassay device.

978-0-9798064-5-2/μTAS 2012/$20©12CBMS-0001

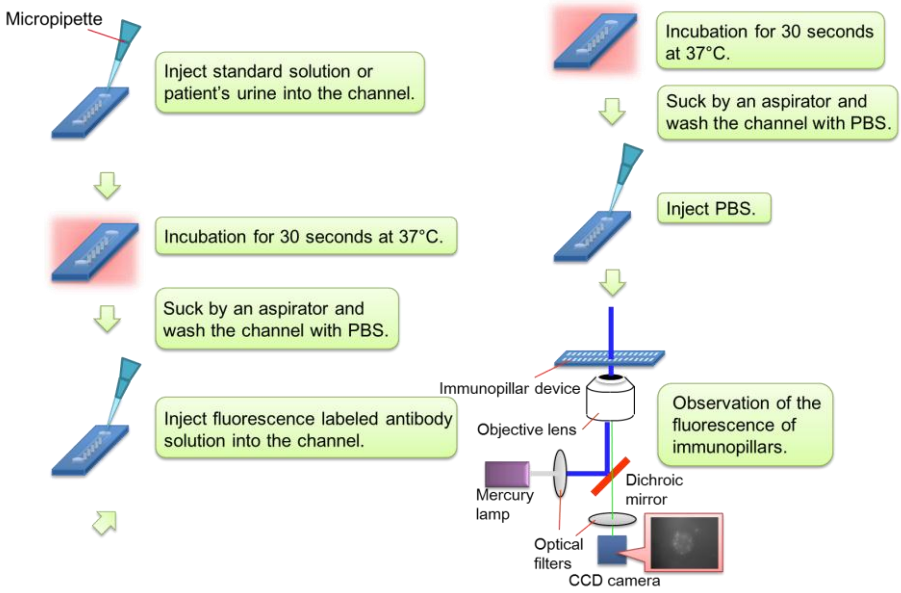

Figure 2: Immunoassay procedure.

ASSAY OPERATION

The procedures of sandwich immunoassay using the immuno-pillar devices are depicted in Fig. 2. We introduced 0.25 μL of the antigen solution or urine sample into the microchannel and incubated for 30 seconds at 37°C. After the incubation, the solution inside the microchannel was sucked by an aspirator and the inside walls of the microchannel and the immuno-pillars were washed 3 times with PBS to remove non-specific binding antigen. Then, we introduced 0.25 μL of the fluorescent-labeled secondary antibody into the microchannel and incubated for 30 seconds at 37°C. After the incubation, the solution inside the microchannel was sucked by the aspirator and the inside walls of the microchannel and the immuno-pillars were washed 3 times with PBS to remove unreacted secondary antibody. Finally, the microchannel was filled with PBS and the fluorescence signal from the immuno-pillars were detected by using an inverted fluorescence microscope (Ti-U, Nikon, Tokyo, Japan) equipped with a CCD camera (EM-CCD, Hamamatsu Photonics, Hamamatsu, Japan) and super high pressure mercury lamp (Nikon, Tokyo, Japan).

RESULTS AND DISCUSSION

In order to measure the concentrations of MCP-1, AGT and L-FABP in the urine samples, we adopted the external standard calibration curve method. The standard curve for each MCP-1, AGT and L-FABP was established with standard solutions. The standard curves with urine data are shown in Fig. 3. The blue and red plots were obtained with standard solutions and human urine samples, respectively. The concentration values of the human urine samples were derived by the conventional microtiter plate method. The limits of detection were estimated as MCP-1: 15 pg/mL=1.72 pM; AGT: 30 pg/mL=378 fM; and L-FABP: 20 pg/mL=1.25 pM, which gave a signal at 3 SDs (standard deviations) above the back ground. We can see that the urine data fit well to each calibration curve. The concentrations in the human urine samples were estimated by horizontally projecting to the least squares line derived from its three nearest blue plots. Then, we carried out the regression analysis of the correlations between the results given by using the immuno-pillar devices and the conventional microtiter plate sandwich immunoassay (Fig. 4). The analysis of the data confirmed that immunopillar devices provide accurate detection of each marker included in human urine and can be utilized widely in the early stage diagnosis of diabetic nephropathy.

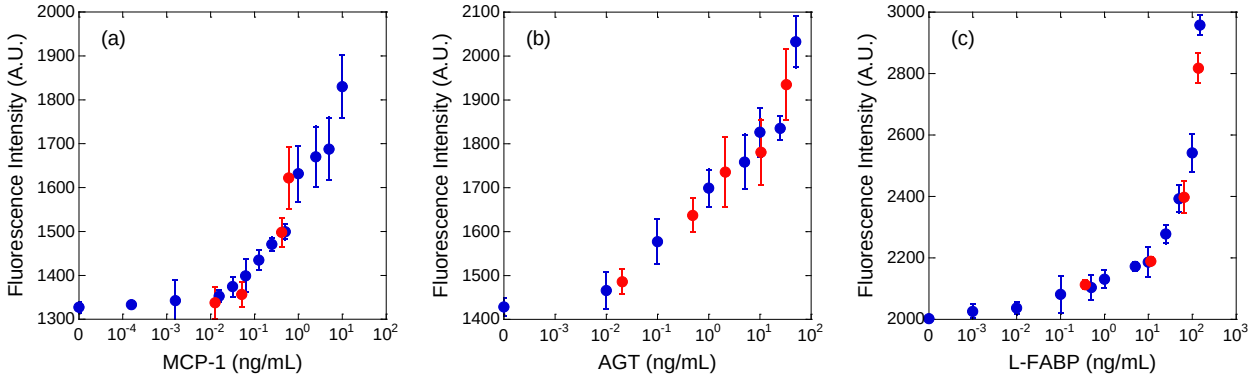

Figure 3: Calibration curves of (a) MCP-1, (b) AGT and (c) L-FABP. The blue and red plots were obtained with standard solutions and human urine samples, respectively.

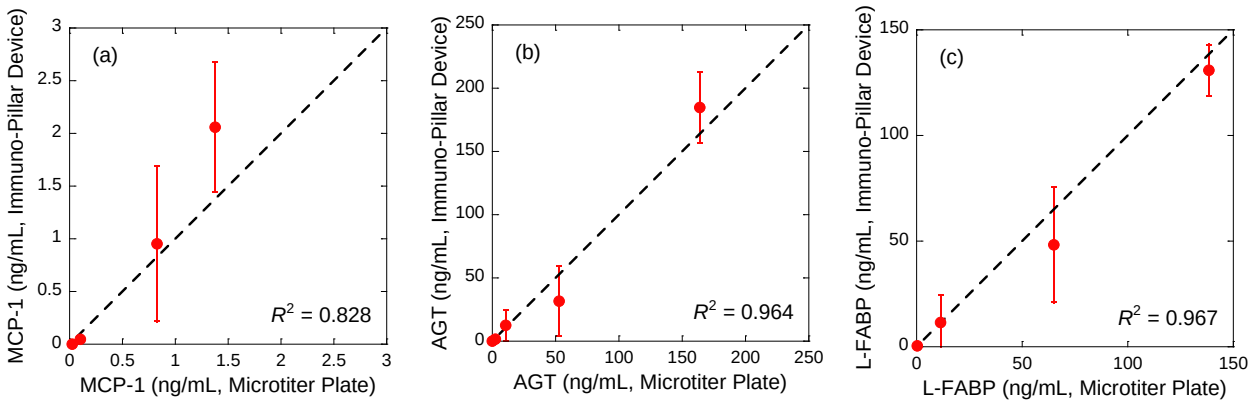

Figure 4: Regression analysis of the correlations between the results given by using immunopillar device and the conventional microtiter plate sandwich immunoassay. Dashed lines indicate the ideal correlation. The values of coefficient of determination (R^2) denote the correlation between the plots and the dashed line.

CONCLUSIONS

Utilizing the second generation immuno-pillar devices, we need only 2 min to achieve immunoassay of urine samples of the patients without any pretreatment and gain higher sensitivity for three important biomarkers of diabetic nephropathy (detection limits of MCP-1: 15 pg/mL=1.72 pM; AGT: 30 pg/mL=378 fM; and L-FABP: 20 pg/mL=1.25 pM). These results are attributable to the higher surface to volume ratio of immuno-pillar containing ten times more number of microbeads and faster migration of biomarkers through larger mesh size of immuno-pillar.

ACKNOWLEDGEMENT

This work was partially supported by Grant-in-Aid for Scientific Research (A) and Suzuken Memorial Foundation.

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