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Author(s)	Mohammadi, Saeed; Busa, Lori Shayne Alamo; Maeki, Masatoshi et al.
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Notes

Rapid Detection of Cat Cystatin C (cCys-C) Using Immuno-Pillar Chips

Saeed Mohammadi,[*1] **Lori Shayne Alamo Busa,**[*1] **Masatoshi Maeki,**[*2] **Reza M. Mohamadi,**[*3] **Akihiko Ishida,**[*2] **Hirofumi Tani,**[*2] **and Manabu Tokeshi**[*2,*4,*5,*6†]

*1 *Graduate School of Chemical Sciences and Engineering, Hokkaido University, Kita 13 Nishi 8, Kita, Sapporo 060–8628, Japan*
*2 *Division of Applied Chemistry, Faculty of Engineering, Hokkaido University, Kita 13 Nishi 8, Kita, Sapporo 060–8628, Japan*
*3 *Department of Pharmaceutical Science, Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Ontario M5S 3M2, Canada*
*4 *ImPACT Research Center for Advanced Nanobiodevices, Nagoya University, Furo-cho, Chikusa, Nagoya 464–8603, Japan*
*5 *Innovative Research Center for Preventive Medical Engineering, Nagoya University, Furo-cho, Chikusa, Nagoya 464–8601, Japan*
*6 *Institute of Innovative for Future Society, Nagoya University, Furo-cho, Chikusa, Nagoya 464–8601, Japan*

We demonstrated a rapid immunoassay for detection of cat cystatin C (cCys-C) which is an important marker for chronic kidney disease in cats, using immuno-pillar chips. The required amount of reagent solution is 200 times smaller than that for the conventional ELISA in the 96-well microplate (0.5 µL *versus* 100 µL). In addition, the total assay time in the proposed method is more than 12 times shorter than in the conventional method (20 min *versus* 240 min). The limit of detection in the new method of 3 ng mL^{-1} is comparable to that of the conventional method (1 ng mL^{-1}) and it is in the clinically relevant range.

Keywords Chronic kidney disease, immunoassay, cystatin C, immuno-pillar chips

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Introduction

Chronic kidney disease (CKD) is widely diagnosed in cats, especially older cats.[1] Several studies have shown that 53% of cats aged 7 years or older and 69 – 81% of cats aged 10 years or older are diagnosed with CKD.[2] Diagnosis of renal dysfunction in the early stage is essential for preventing or delaying additional renal damage.[3,4] A direct measurement of glomerular filtration rate (GFR) is considered the gold standard for evaluating kidney function.[5] However the measurement is expensive and time-consuming.[6] Hence, development of an inexpensive, user-friendly and rapid technique to detect minor renal dysfunction in cats is desired. Despite various techniques that have been developed to explore symptoms of renal failure in human serum, detecting these failures in cats still has not attracted much attention.[7] The causes of CKD are heterogeneous and rarely identified; however the commonly used indirect GFR markers, serum creatinine (sCr) and blood urea nitrogen (BUN), are not sufficiently sensitive or specific to detect early renal dysfunction.[2] The serum concentrations of these markers increase when approximately 75% of the functional renal mass is lost.[5] These markers, especially BUN, are influenced by non-renal factors, such as age, diet, hydration status, and muscle mass.[5] Cystatin C (Cys-C) is a low molecular weight (13 kDa) cysteine proteinase inhibitor that is produced in cells at a constant rate and is part of the protein catabolism network.[8,9] Cys-C has low intra-individual variability, no plasma protein binding, no tubular secretion, no tubular reabsorption without catabolism, and no extrarenal clearance.[10] These properties are ideal for endogenous GFR marker applications.

On the other hand, microfluidic devices are a technology to integrate various assays on a single chip for low-cost, user-friendly, and rapid detection of markers.[11,12] We previously developed immuno-pillar chips as a rapid, inexpensive, easy-to-use, and highly sensitive chip applicable for multiplex assays.[13-15] Here we report the detection of cat Cys-C (cCys-C) using the immuno-pillar chips. The antibody-immobilized microbeads assembled in these pillars create efficient reaction sites for capturing cCys-C.

Experimental

Fabrication of the immuno-pillar chips and procedure for cCys-C assay

Figure 1 illustrates the fabrication procedures for the micro immuno-pillars in the microchannels.

First, the beads that were used to form the immuno-pillars were prepared. An affinity bead kit (Catalog No. BS-X9905) was purchased from Sumitomo Bakelite Co., Ltd., Osaka,

† To whom correspondence should be addressed.
E-mail: tokeshi@eng.hokudai.ac.jp

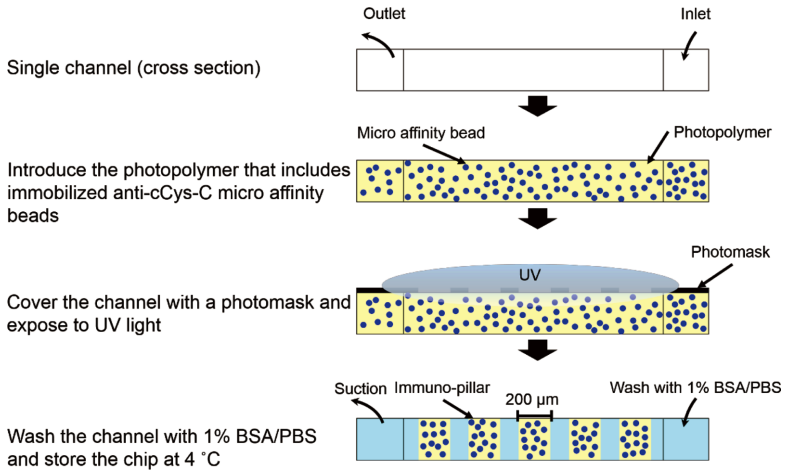

Fig. 1 Fabrication steps for the photopolymerized micro immuno-pillars.

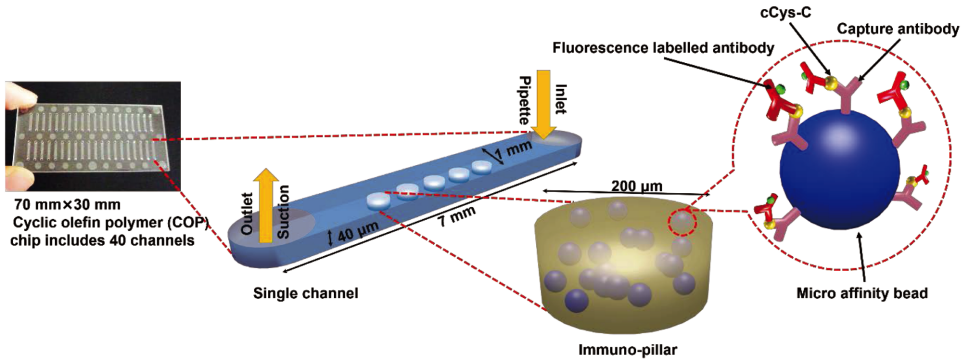

Fig. 2 Schematic of cCys-C assay in a channel of the immuno-pillar chips.

Japan.[15] A 10-mg amount of the affinity beads (5 µm in diameter) and 150 µg mL^{-1} anti-cCys-C (mouse monoclonal IgG) in the coupling buffer were transferred to a microcentrifuge tube and incubated for 4 h at 37˚C in a rotary shaker. Then, the microtube was centrifuged at 5000*g* for 60 s at 25˚C and the supernatant was discarded. The beads were washed three times with the wash buffer (0.01% Tween 20/PBS) and centrifuged as mentioned above to remove the washing solution. Then 500 µL of the blocking buffer was added to the beads and this was incubated for 1 h at room temperature. After that, the microtube was centrifuged at 5000*g* for 60 s at 25˚C and the supernatant was discarded. The beads were washed three times with a PBS buffer as mentioned above. Finally, the washing solution was discarded and the beads were dried for 1 h at 37˚C.

Next, the microchannels in which the immuno-pillars were to be fabricated were prepared. Cyclic olefin polymer (COP) chips, each with 40 microchannels, were purchased from Sumitomo Bakelite Co., Ltd. A photocrosslinkable prepolymer (Catalog No. ENTG3800) and a photoinitiator (Catalog No. PIR-1) were purchased from Kansai Paint Co., Ltd, Osaka, Japan. A mixed solution of a PBS buffer, the photocrosslinkable prepolymer, and the photoinitiator was prepared in the volume ratio of 150:25.5:12, respectively. Then, 125 µL of this solution was added to the dried beads, followed by gentle mixing. The suspension of the beads was introduced into the microchannels on the COP chip. Next, the chip was covered with a photomask that had five open areas where pillars (200 µm in diameter) were made in each microchannel and the chip was irradiated with ultraviolet light for 60 s. After removing the photomask, the channels with the immuno-pillars were washed with 1% BSA/PBS and the chip was stored at 4˚C prior to use.

The procedure for the cCys-C assay was as follows. All the channels with the micro immuno-pillars were washed five times with 0.01% Tween 20/PBS. Then, seven different concentrations of cCys-C (Nipro Co., Ltd., Osaka, Japan) from 0 to 50 ng mL^{-1} were introduced into the channels separately and each solution was incubated for 10 min at room temperature. Afterwards, the channels were rinsed with PBS buffer five times. Fluorescence-labeled anti-cCys-C (mouse monoclonal IgG) was prepared using the Zenon® Alexa Fluor® 488 rabbit IgG labeling kit (Thermo Fisher Scientific Co. Ltd., Yokohama, Japan). This anti-cCys-C solution was introduced into the channels and was incubated at room temperature for 10 min in the dark. Finally, the channels were rinsed with a PBS buffer five times. Fluorescence images were captured by a fluorescence microscope (ECLIPSE Ti-U, Nikon Co., Ltd., Tokyo, Japan) equipped with a CCD camera (ORCA-R2, Hamamatsu Photonics K. K., Hamamatsu, Japan) and the intensity was measured using AquaCosmos software (Hamamatsu Photonics K. K.). Figure 2 shows a schematic of cCys-C assay in a microchannel with the immuno-pillar chips.

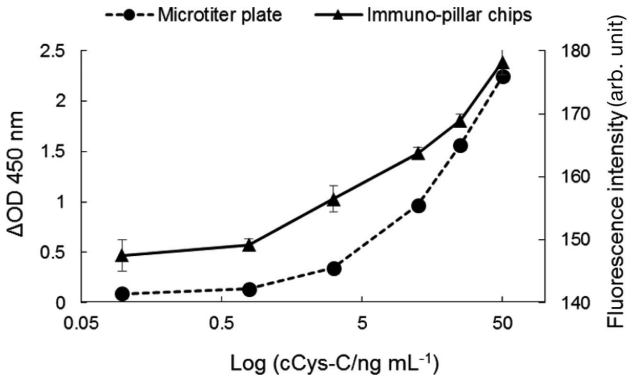

Fig. 3 Standard curves for cCys-C using the immuno-pillar chips (solid line with right Y-axis) and microtiter plate (dashed line with left Y-axis).

Control tests in microtiter plate

A cCys-C ELISA kit from Nipro Co., Ltd. was used, and a control test in a microtiter plate was carried out based on the manufacturer's instructions. First, 100 µL of cCyc-C solutions in concentrations from 0 to 50 ng mL^{-1} were pipetted into a 96-well microtiter plate, which was pre-coated using anti-cCys-C antibody. Then the plate was incubated at 37°C for 1 h. Afterwards, each well of the plate was rinsed seven times with 350 µL of the washing buffer. Next, 100 µL of the horseradish peroxidase-labeled anti-cCys-C antibody was pipetted into each well and the mixture was incubated for 30 min at 37°C. Then, each well was rinsed nine times with 350 µL of the washing buffer. Afterwards, 100 µL of the substrate solution consisting of 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide was pipetted into each well and incubated for 30 min at room temperature in the dark. Finally, the kit stop solution was pipetted into each well (100 µL) and the absorbance at 450 nm was measured using a microplate reader (Model Sunrise-Basic Tecan, Tecan Japan Co., Ltd., Kawasaki, Japan) within 30 min.

Results and Discussion

Detection of cCys-C, as mentioned earlier, has great potential for application to the diagnosis of renal dysfunction in cats at an early stage. We carried out sandwich immunoassays of cCys-C using our immuno-pillar chips and compared the results with those obtained with a conventional ELISA using a 96-well microtiter plate. Each pillar contained about 33000 beads which provided a large surface area to incubate the capture antibody and detect cCys-C with high sensitivity. After incubation with the fluorescence-labeled antibody, we measured the fluorescence intensity. To measure the fluorescence intensity, a circular area of 200 µm diameter was drawn around each pillar using Aqua Cosmos software and average fluorescence intensity was measured. For each of the seven concentrations of cCys-C from 0 to 50 ng mL^{-1}, the fluorescence intensity of 15 pillars was measured. Standard curves of cCys-C using our immuno-pillar chips and the 96-well microtiter plate are shown in Fig. 3. The standard curves had different logistic gradients because the detection methods were different. The fluorescence intensity increased with increasing cCys-C concentration in the immuno-pillar chips and had good reproducibility. The limits of detection (LODs) were calculated as three times the standard deviation (SD) of the signal from a blank pillar with zero cCys-C concentration. The LODs for cCys-C using immuno-pillar chips and the 96-well microtiter plate were 3 and 1 ng mL^{-1}, respectively. The higher LOD for the former was because of the higher background signal. However, the cut off value is 1.05 µg mL^{-1} which means the immuno-pillar has enough sensitivity.[7] The absorbance in a 96-well microtiter plate also increased with increasing concentration of cCys-C.

Table 1 Comparison of the immuno-pillar chips and the 96-well microtiter plate for cCys-C detection

Item	Immuno-pillar chips	96-well microtiter plate
Total assay time/min	20.0	240
Volume of reagent/µL	0.5	100
LOD/ng mL^{-1}	3	1
Cost/$ per assay	2[a]	25

a. The price of the chip and the reagents (including chemicals and antibodies) were estimated to be $1.00 each.

A comparison of our chip assay and the conventional ELISA assay is made in Table 1. In this study, the total assay time included the times for the first and second incubations, washing, and detecting steps; for our chip, it was only 20 min while it was 240 min with the 96-well microtiter plate. In addition, reagent consumption was significantly lowered from 100 µL in the 96-well microtiter plate to 0.5 µL in the immuno-pillar chips. However, improvement of the LOD in the immuno-pillar chips (3 ng mL^{-1}) requires further consideration; the 96-well microtiter plate had a LOD of 1 ng mL^{-1}. In conclusion, we have developed a new microfluidic assay for detection of cCys-C that is faster, less expensive and requires use of much less reagent. For example, the cost for the current assay can be as much as 10 times lower than that of the conventional 96-well microtiter plate. This method can be further developed for veterinary point of care diagnosis of renal failure in cats.

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