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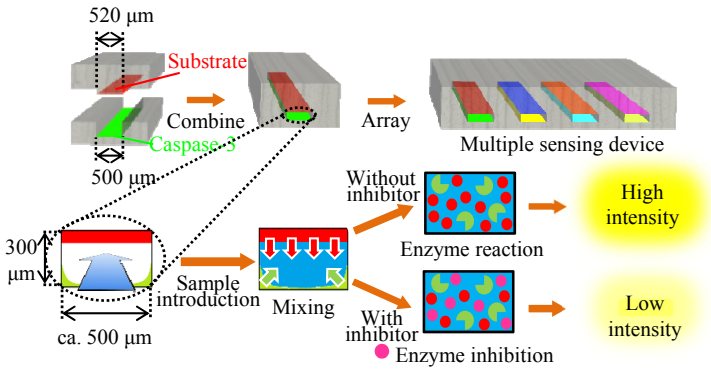

Caspase-3 inhibitor assay was successfully integrated into ”Single-Step” by solving the problem of low-activity enzyme immobilization by using a combinable poly(dimethylsiloxane) capillary (CPC) sensor.

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Efficient Immobilization of Enzyme and Substrate for Single-Step Caspase-3 Inhibitor Assay Using Combinable PDMS Capillary Sensor Array

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The caspase-3 inhibitor assay, which is important for drug development was successfully integrated into a "single step" by solving the immobilization problem of enzyme with low activity. Here we used the soluble polyethylene glycol (PEG) coatings for enzyme immobilization to the concave-shaped PDMS surface, and substrate immobilization to the convex-shaped PDMS surface. Then these PDMS structures were conbined to form capillary-structure, which we call, a combinable poly (dimethylsiloxane) (PDMS) capillary (CPC) sensor, enabling simultaneous immobilization of two different reactive reagents such as enzymes and fluorescent substrates. The present sensor was a disposable use, and the fluorescence response was simply obtained by introducing the sample solution by capillary action. The caspase-3 activity was able to be maintained at approximately 90% under -80 ℃ storage condition even after 5 months. Importantly, the total reaction time was reduced from an hour to 3-5 min, and simultaneous acquisition of multiple data sets required to determine IC_{50} value was also successfully achieved using the CPC sensor array.

1.Introduction

Enzymes are very important targets for drug development against various diseases, such as Alzheimer, cancer, and AIDS [1-3]. The identification of specific inhibitors for given enzymes from many compounds libraries is essential at the beginning of drug development; therefore, a rapid and low-cost enzyme inhibitor assay is needed [4]. To date, many enzyme inhibitor assays have been developed. For example, Clausell-Tormos *et al*. reported a droplet-based high-throughput system with an enzyme immobilized monolith micro-reactor [5], whereas Garcia *et al*. developed a multi laminar flow-based enzyme inhibitor assay [6]. Recently, Cai *et al*. demonstrated that a microfluidic chip-based system was capable of generating droplet array with a large scale concentration gradient by coupling the flow injection gradient technique with droplet-based microfluidics [7], and Zeng *et al*. reported a real-time colorimetric enzyme inhibition assay using functionalized gold nanoparticle [8]. While these previously reported assays are high-throughput, the operation difficulties, system complexity, and time-consuming functionalization procedures of nanoparticles are still major problems.

Since several diseases, including cancer, inflammation, and Alzheimer's, are related to apoptosis, alterations of the apoptotic process might offer important information regarding the treatment of these diseases. The caspase family proteins are key components of the cellular apoptic pathway and therefore have become important targets for drug discovery. In the caspase cascades, upstream caspases (initiator caspases) are first activated via the interaction with apoptosis-promoting proteins. Once activated, initiator caspases process and activate one or more downstream caspases (effector caspases). Activated effector caspases subsequently cleave specific sets of cellular proteins, leading to apoptotic cell death [9, 10]. Among all the effecter caspases, caspase-3 is a converging point of the apoptotic pathways and therefore is particularly important for drug discovery [11].

Previously, our group has been focusing on the development of functionalized capillary sensors and related microfluidic devices [12-24]. Functionalized capillary sensors have the following 3 major advantages. First, reagent and sample volumes are very small (typically with a 100-fold smaller than those used in microplates (1.5 μL/cm of CPC length).). Second, assays can be simply attained only by introducing samples via capillary action. Third, a simple arraying of various capillaries allows multiple sensing. However, a single-step enzyme inhibitor assay has been difficult to achieve using commercially available capillaries, because it is necessary to immobilize enzymes and substrates inside the same capillary. To solve this issue, we recently developed a new capillary-type sensor called the combinable poly (dimethylsiloxane) (PDMS) capillary (CPC) sensor. Specially, our CPC sensor, prepared by combining convex and concave PDMS substrate, enables the immobilization of different reactive reagents (such as enzymes and substrates or antigens and antibodies) separately in the same capillary without contamination, thus CPC sensors allowed single-step enzyme inhibitor assay for disposable use. As a proof of concept, a single-step trypsin inhibitor assay was successfully performed using the Layer-by-Layer (LBL) method for enzyme immobilization [25].

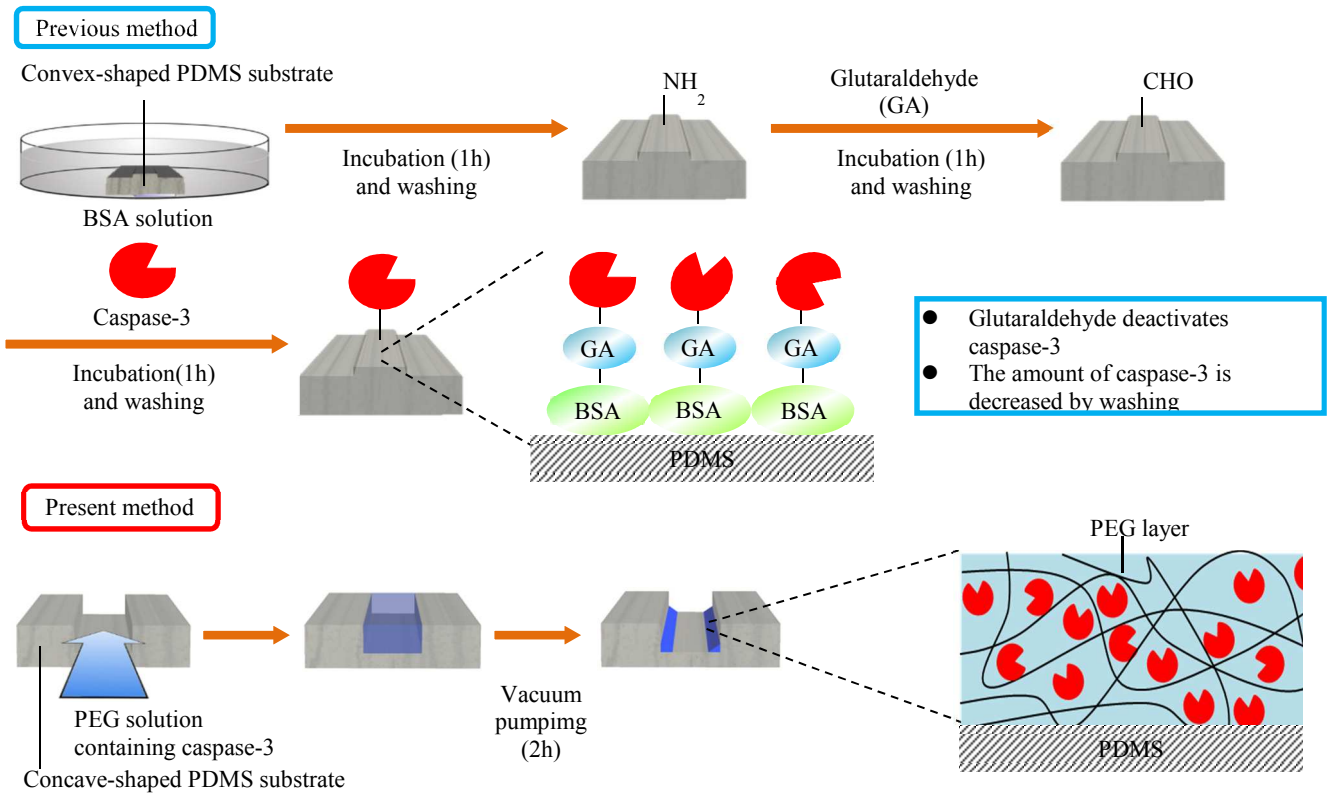


Fig. 1:Comparison between previous and present methods

To further improve the disadvantages of the short light path length of capillaries and the hydrophobicity of PDMS, an enhancement of sensitivity and the facilitation of sample introduction by bulk and surface modifications in CPC sensors were achieved [26]. Recently, this CPC sensor was successfully applied to the neuraminidase inhibitor assay using the glutaraldehyde method for enzyme immobilization, thereby eliminating complicated procedures of controlling pH condition [27].

To apply CPC sensor to drug discovery for cancer or Alzheimer's, the development of a caspase-3 inhibitor assay system is indispensable. However, due to the difficulty of immobilizing enough amounts of enzymes, conventional enzyme immobilization methods such as LBL and glutaraldehyde methods could not be used for caspase-3 immobilization. Therefore, it is important to develop a new immobilization method that enables the immobilization of enough amounts of enzymes without losing enzyme activity, especially for low-activity enzymes like caspase-3. The aim of this study is to develop a novel method of enzyme and substrate immobilization for the single-step caspase-3 inhibitor assay. We focused on the use of a soluble polyethylene glycol (PEG) coating for enzyme immobilization (Fig. 1) [17,18], which is a dried mixture of PEG and enzyme, and is expected to avoid adsorption of enzyme to the PDMS surface, avoid aggregation of enzymes which sometimes the cause of activity decrease [28], and promote the diffusion of enzyme when sample solution was introduced. Moreover, since the PEG has been considered as an enzyme stabilizer [29, 30], long term stability is also expected. Furthermore, since the present method involves the introduction of PEG solution containing enzymes followed by drying up procedure (Fig.1), loading amount of enzyme can be easily controlled. Fig. 2 shows the general concept of this newly developed CPC sensor array. When the sample inhibitor solution is introduced into the capillary by capillary action, enzyme and the fluorescent substrates are released from the capillary wall and spontaneously mixed with the inhibitor. Here, we demonstrated the preparation of the CPC sensor array and its application to the single-step caspase-3 inhibitor assay.

2. Experimental Section

2.1 Chemicals

Poly (ethyleneglycol) (PEG; Mw, 20,000) and glutaraldehyde solution (25%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). PDMS pre-polymer (SILPOT 184) and curing agent (SILPOT184 CAT) were acquired from Dow Corning Toray Co., Ltd. (Tokyo, Japan). Poly [dimethyl siloxane-comethyl-(3-hydroxypropyl)-siloxane]-graft-PEG methyl ether (PDMS-PEG) and caspase-3 were obtained from Sigma-Aldrich (St Louis, MO, USA). Fluorescent substrates, Ac-Asp-Glu-Val-Asp-MCA (Ac-DEVD-MCA) and inhibitors, Ac-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO) were purchased from Peptide Institute, Inc. (Osaka, Japan).

2.2 Fabrication of CPC sensor array

"Convex"- and "concave"-shaped PDMS substrates were

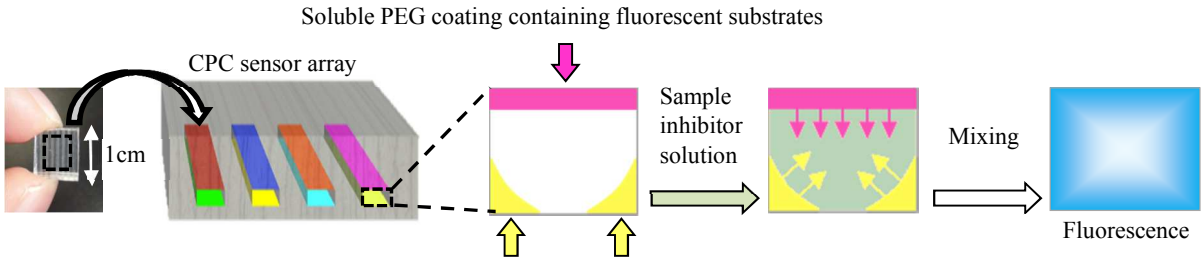


Fig 2: General concept for the immobilization of different reagents inside the CPC sensor array without contamination

prepared as described previously [25]. Convex-shaped PDMS substrate had a width of 520 μm and a height of 200 μm, whereas concave-shaped PDMS substrates had a width and a depth of 500 μm. When two types of PDMS substrates were combined, the slight difference between their sizes improved adhesion, thereby preventing solution leakage. A CPC sensor array was prepared only by combining 2 types of PDMS substrates and the channel had a width of approximately 500 μm and a height of 300 μm.

2.3 Immobilization of enzymes to concave-shaped PDMS substrates

A 2.5-μL cocktail solution of HEPES buffer (pH 7.4) containing caspase-3 (2.0×10^{-7} M), PEG 20000 (1mg/mL), and PDMS-PEG (1 μL/mL), was introduced into each concave shaped PDMS substrate (length, 10 mm), followed by dried for 2 h by vacuum pumping.

2.4 Immobilization of fluorescent substrates to convex-shaped PDMS substrates

Solutions of 240 mg/mL PEG 20000 in methanol containing fluorescent substrates (10^{-2} M), PDMS-PEG (100 μL/mL), and DMSO (100 μL/mL) were spin-coated on glass plates. Convex-shaped PDMS substrates after plasma treatment (2 min) were stamped to glass plates, peeled off and dried to form PEG coatings containing fluorescent substrates.

2.5 Preparation of a CPC sensor for the caspase-3 inhibitor assay

A CPC sensor array was prepared by combining concave- and convex-shaped PDMS substrates containing immobilized enzymes and fluorescent substrates, respectively. Inhibitor solutions at various concentrations (1.5 μL/cm of CPC length) were introduced into capillaries of the CPC sensor array via capillary action, and both ends of capillaries were sealed using a manicure to prevent evaporation. Fluorescence was evaluated by fluorescence microscopy (Multi Viewer System VB-S20; Keyence Corporation, Osaka, Japan) and analyzed by Image J (version 1.43; National Institutes of Health, Bethesda, MD, USA).

3. Results and Discussion

3.1 Evaluation of the soluble PEG coating on concave- and convex-shaped PDMS substrates

The fluorescent dye, 7-amino-4-methyl coumarin (AMC), was used to visualize soluble PEG coatings instead of caspase-3 or fluorescent substrates. Fig. 3 shows soluble PEG coatings on the concave-shaped PDMS substrate. A uniform PEG coating along

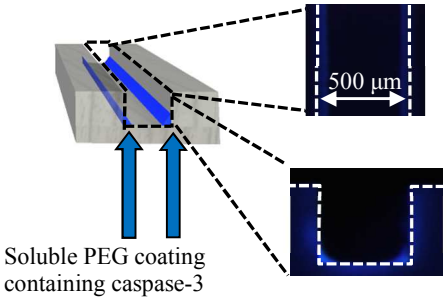


Fig 3: Immobilization of caspase-3

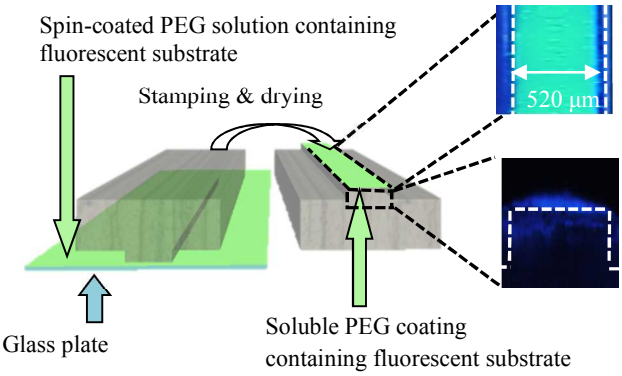


Fig 4: Immobilization of substrate

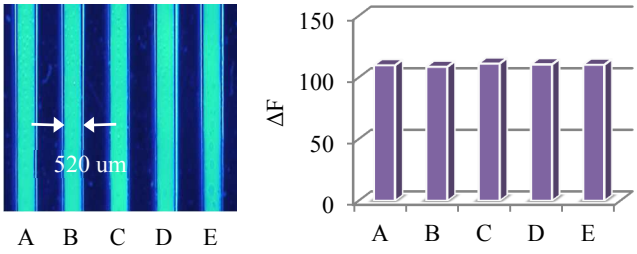


Fig 5:Uniformity of the PEG coating by stamping method

the channel was observed. This immobilization method via physical adsorption was very easy and did not require washing procedure; therefore, no enzyme or enzyme activity was lost during surface coating. Fig. 4 indicates that a uniform coating of soluble PEG on the convex-shaped PDMS substrate was successfully achieved by stamping. Fig. 5 further shows that the error of the amount of immobilization was very small and that RSD was also less than 5%. These results enable the mass production of CPC sensor arrays by slicing a long capillary array with immobilized enzymes and fluorescent substrates into small pieces.

3.2 Enzyme reaction profile of the CPC sensor array

A CPC sensor with the length of 1 cm was prepared as described above. While native PDMS has the hydrophobic property, the PEG coating increased the water wettability of PDMS substrates, thereby making sample introduction via capillary action possible. Fig. 6 shows a representative fluorescence image and an enzyme reaction profile after HEPES buffer solutions (pH 7.4) were introduced into CPC and incubated for 15 min. The fluorescence response, which was difficult to observe with the LBL or glutaraldehyde method (see supporting information), was successfully detected. The use of the LBL or glutaraldehyde method also decreased the amount of enzymes due to the washing procedure of immobilization. In addition, caspase-3 may be deactivated by glutaradehyde because an aldehyde group is involved in the inhibition of caspase-3 activity (i.e., Ac-DEVD- CHO). The present method, on the other hand, was able to maintain sufficient amounts of immobilized enzymes without losing enzyme activity, owing to the biocompatible PEG coating. Importantly, the total analysis time was shortened from an hour reported in the literature [31] to 3-5 min. Since this inhibition assay involves the dissolution and reaction of fluorescent substrate and caspase-3 inside the CPC to form AMC, rapid mixing in small-sized capillaries might contribute to the reduction of reaction time.

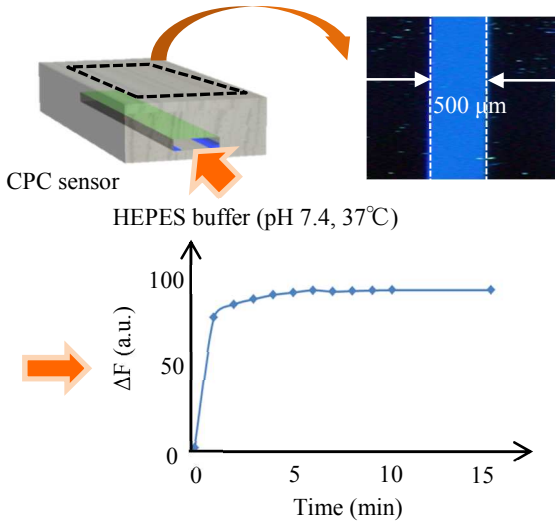


Fig 6: Enzyme reaction profile

3.3 Caspase-3 inhibitor assay

Here, we carried out the single-step caspase-3 inhibitor assay by introducing samples via capillary action. The use of the CPC sensor eliminates conventional analytical operations, such as premixing of enzymes, fluorescent substrates, and inhibitors. In Fig. 7, a typical inhibition curve for the CPC shows inhibitor concentration dependence when various concentrations of the inhibitor solutions (pH 7.4) were introduced. The consumption of inhibitors was only 1.5 μL per 1 cm capillary channel, which was significantly reduced (about 100-fold) compared to the amount used in microtiter plates. Fig. 8 shows the stability of immobilized caspase-3 on the PDMS substrate when stored in the deep freezer (-80℃). The fluorescence intensities were obtained after introducing 10^{-4} M fluorescent substrate into the CPC sensor array with immobilized caspase-3. The horizontal axis shows the storage period after caspase-3 immobilization. Even after 150 days storage, the caspase-3 activity was still maintained at 90%. Reason of this stability is not clear, but since caspase family works as multimeric form [10], PEG may work as a scaffold to keep stable structure during the storage. It should be noted that the enzyme immobilization without PEG (i.e. direct adsorption of enzyme to the PDMS surface) resulted in slight decrease of fluorescence response (Fig.9). In Fig.9, one PDMS channel was filled with caspase-3 solution (0.2 μM) containing PEG 20000 (1 mg/mL), and the other PDMS channel was filled with the same solution but without PEG 20000. In each solution, PDMS-PEG (1 μL/mL) was included. After drying, fluorescent substrate solutions (10^{-4} M) were introduced into both channels. Just after drying, immobilization without PEG resulted in ca. 10% weaker fluorescence than the case of using PEG, indicating that some amount of caspase-3 is already not active (Day 0). This may be attributed to the enzyme activity loss by conformational change of enzyme associated with adsorption to the PDMS, or aggregation [28]. After storage under -80 ℃ for two days, immobilization without PEG exhibited weaker fluorescence (Day 2). Since the storage of caspase-3 using PEG under -80 ℃ resulted in 5 month stability (Fig.8), usefulness of PEG for immobilization of caspase-3 was confirmed. According to these results, the long-term storage of the CPC sensor array may be possible for the caspase-3 inhibitor assay. Taken together, these results indicate that the single-step caspase-3 inhibitor assay using the CPC sensor array was successfully achieved and may be expected to promote rapid and low-cost drug development.

4. Conclusions

The single-step caspase-3 inhibitor assay using the CPC sensor array was successfully developed. To ensure the presence of sufficient amounts of caspase-3 and prevent enzyme activity loss, caspase-3 was immobilized on the concave-shaped PDMS substrate via physical adsorption using the soluble PEG coating. The fluorescent substrates for caspase-3, on the other hand, were immobilized on the convex-shaped PDMS substrate by stamping. Using the CPC sensor prepared by combining these two types of PDMS substrates, the fluorescence response, which is difficult to observe with the LBL or glutaraldehyde method, was successfully

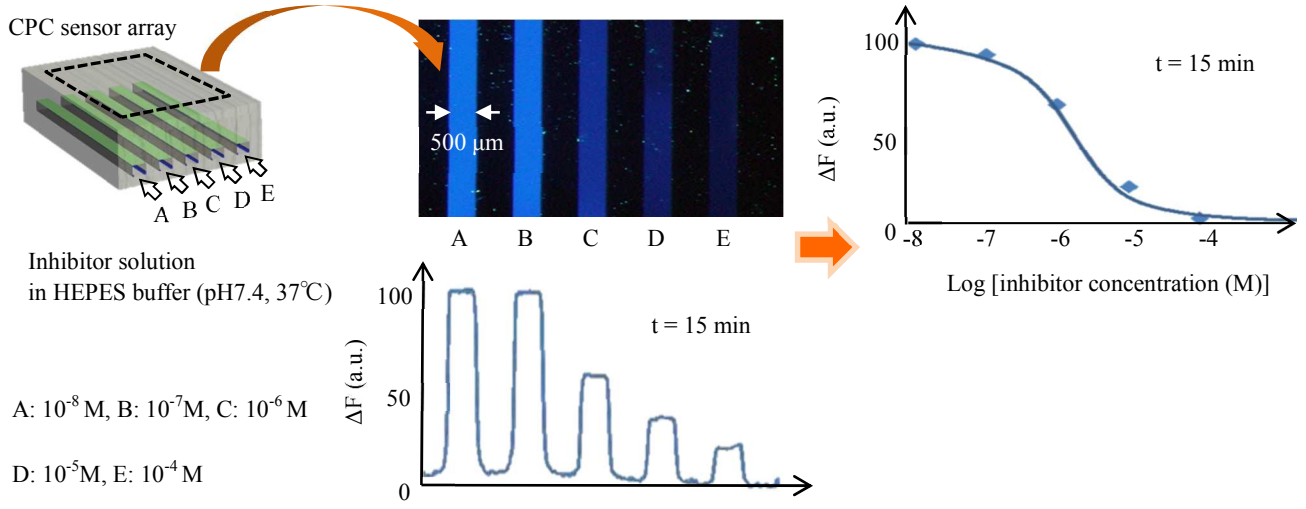
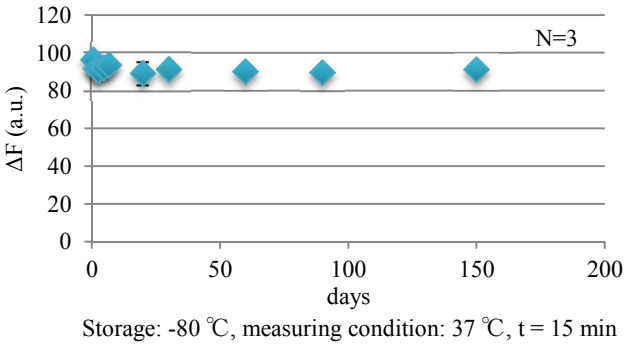


Fig 7: Caspase-3 inhibitor assay using CPC sensor array



Storage: -80 ℃, measuring condition: 37 ℃, t = 15 min

Fig 8: Stability of caspase-3 activity

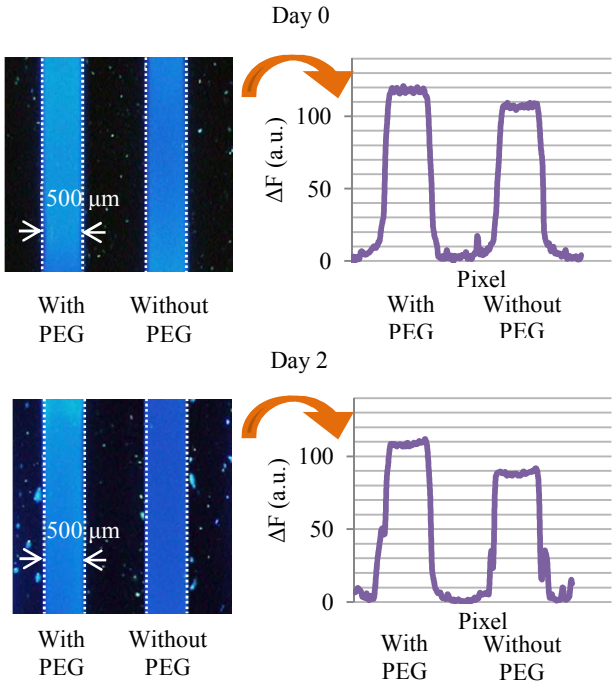


Fig 9: Effect of PEG on caspase-3 activity

detected, and the total reaction time was shortened from an hour to 3-5 min when buffer solution were introduced into the CPC sensor. Next, the caspase-3 inhibitor assay was carried out using the CPC sensor array. The inhibition curve, which was plotted by simultaneously acquiring multiple sets of data, indicates that the fluorescence intensity was dependent on inhibitor concentrations (10^{-8}-10^{-4}M). In addition, the consumption of inhibitor samples was significantly reduced (about 100-fold), and the CPC sensor array was stable for at least 5 months in freezer. In conclusion, our results indicate that the CPC sensor array may not only be applied for various types of enzyme inhibitor assays but also become a useful analytical tool for the research in the field of drug screening.

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