Single-Step Trypsin Inhibitor Assay on a Microchannel Array Device Immobilizing Enzymes and Fluorescent Substrates by Inkjet Printing

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Abstract

In this study, we report a single-step trypsin inhibitor assay on a microchannel array device immobilizing enzymes and substrates by inkjet printing. The microdevice is composed of a poly(dimethylsiloxane) (PDMS) microchannel array that immobilizes trypsin and fluorescent substrates as reactive reagents at the two bottom corners of a microchannel. Inkjet printers allow simple, accurate, and position-selective immobilization of reagents as nanoliter spots. Therefore, plural reactive reagents, such as enzymes and substrates, can be separately immobilized at different positions in the same microchannel without mixing, allowing single-step operation by simply introducing a sample solution through capillary action. Furthermore, reproducible fabrication and mass production of the device could be expected. In this study, the efficiency of an acidic solution as a spotting agent for protease immobilization to prevent the decrease in fluorescence intensity was confirmed. Additionally, single-step trypsin inhibitor screening was performed using three inhibitors. Finally, we investigated the storage stability of the device and confirmed that it remained stable for at least 10 days.

Keywords: enzyme inhibitor assay, inkjet printing, microchannel, poly(dimethylsiloxane), single-step
Introduction

Enzyme inhibitor assay is an essential tool for drug discovery.\textsuperscript{1} It usually involves the analysis of a significant quantity of bioactive compounds in large libraries. Therefore, time-saving, high-throughput, and simple techniques using low sample volumes are required.\textsuperscript{1–3} To achieve these objectives, several enzyme inhibitor assay-related approaches have been reported.\textsuperscript{4–13} In enzyme inhibitor assays, high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, and capillary electrophoresis (CE) are widely used as analytical methods.\textsuperscript{14} The HPLC-UV methods\textsuperscript{15–17} require a complex operation, long elution time, and a large volume of organic solvents. However, electrophoretic separation-based CE allows high-efficiency separation; multiple enzyme inhibitor assay methods using CE have thus been reported.\textsuperscript{1,3,14,18–21} However, CE requires large equipment and involves multi-step reactions. Additionally, fluorescence or colorimetric assays using microplate have been reported.\textsuperscript{22–24} However, these assays also require complex operation and large amounts of reagents and samples.

In contrast, our group developed a reagent-release capillary sensor array by combining two poly(dimethylsiloxane) (PDMS) substrates coated with different reactive enzyme inhibitor assay reagents.\textsuperscript{25–27} These devices enable the performance of a single-step assay based on sample introduction by capillary action, low sample volume, and multiple detection. However, since the reagent immobilization steps were carried out layer-by-layer, glutaraldehyde crosslinking, or stamping, it is difficult to specify the quantity of reactive reagents immobilized on a substrate. Nevertheless, we have recently developed a single-step, inkjet printing-based bioassay microdevice that can immobilize two reactive reagents independently on a single PDMS microchannel.\textsuperscript{28,29} Inkjet printers allow the accurate and position-selective immobilization of reagents as nanoliter spots, enabling simple and reproducible fabrication and mass production.
In this study, we propose the application of inkjet printing to a single-step trypsin inhibitor assay as a demonstration. We constructed a trypsin inhibitor assay microdevice through the immobilization of trypsin and fluorescent substrates at the two bottom corners of a microchannel (Fig. 1), that allowed single-step operation by simply introducing an inhibitor sample solution. Working principle is the followings. In the absence of trypsin inhibitors in the sample solution, trypsin hydrolyzes fluorescent substrates to form fluorescent products such as 7-amino-4-methylcoumarin (AMC); however, in the presence of trypsin inhibitors, AMC formation is suppressed due to the inhibition of trypsin activity, thus inhibition efficiency can be evaluated by the difference in fluorescence. To demonstrate this concept, we investigated enzyme immobilization conditions, single-step enzyme inhibitor screening, and storage stability of the device.

**Experimental**

*Preparation of the trypsin solution and fluorescent substrate solution for inkjet printing*

To increase the dissolution of the immobilized reagent when a sample solution is introduced into the final device, we used trehalose (HAYASHIBARA Co., Ltd., Okayama, Japan) as an additive for inkjet-printing solutions.\(^{28,29}\) We prepared the trypsin solution by mixing 280 μL of 50 μg/mL trypsin (Sigma-Aldrich, St. Louis, MO, USA) solution in 1 mM HCl aq. (pH = 3), 52.5 μL of 400 mg/mL trehalose solution, and 17.5 μL of water. Then, we obtained trypsin (40 μg/mL) containing 6% (w/v) trehalose in 0.8 mM HCl aq. To prepare the fluorescent substrate solution, we mixed 170 μL of 10⁻⁴ M aqueous solution of the fluorescent substrate (Boc-Gln-Ala-Arg-MCA, PEPTIDE INSTITUTE, INC., Osaka, Japan) in 10 vol% dimethyl sulfoxide (DMSO) and 42.5 μL of 400 mg/mL trehalose. Finally, we obtained the fluorescent substrates (8 × 10⁻⁴ M) containing 8% (w/v) trehalose in 8 vol% DMSO.
Fabrication of the single-step trypsin inhibitor assay microdevice using inkjet printing

To fabricate the single-step trypsin inhibitor assay microdevice, we prepared the PDMS microchannel arrays (channel width: 500 μm, channel depth: 500 μm) following a previously described method. Next, we dispensed the trypsin and fluorescent substrate solution independently as droplets (~28 nL each) at the two bottom corners of a microchannel using an inkjet printer (LaboJet-1000P, Microjet Corporation, Nagano, Japan). In that case, amounts of immobilized fluorescent substrates and enzymes in single droplet are calculated to be 2.2 fmol and 1.1 fg, respectively. The fabricated microdevice was dried at 15 °C for 1–2 days. Aluminum foil was used to cover the microdevice during the drying process to provide shade. Finally, the microdevice was covered by a cover glass to prevent evaporation of the sample solution during the measurement.

Measurement

The sample solutions of 10^{-8}–10^{-2} M leupeptin (PEPTIDE INSTITUTE, INC., Osaka, Japan) in 0.1 M Tris-HCl buffer (pH = 7) were introduced into the microdevice. Then, the fluorescence of the microchannel was measured for 30 min using a fluorescence microscope (VB-7010, Keyence, Osaka, Japan). To prevent evaporation of sample solution, both ends of the microchannel were sealed by PDMS prepolymer immediately after starting the measurement. The final concentrations of trypsin, fluorescent substrates, and trehalose were 5 μg/mL, 10^{-4} M, and 1.75% (w/v), respectively.
Results and Discussion

Initially, conditions of trypsin immobilization were investigated. To evaluate the activity of immobilized trypsin, a trypsin (8 μg/mL) solution containing 8% (w/v) trehalose in 0.08 M Tris-HCl aq. (pH = 7) was used as a spotting agent for immobilization by inkjet printing. After drying at 15 °C for 1–2 h, the 10⁻⁴ M fluorescent substrate solution in 0.1 M Tris-HCl buffer (pH = 7) was directly introduced into the microchannel (the final trypsin and trehalose concentrations were 1 μg/mL and 1% (w/v), respectively); however, no fluorescence was observed. This might be caused by the autolysis of trypsin during the immobilization and drying processes. Therefore, 0.8 mM HCl aq., a storage solution recommended by the manufacturer, was used as a solvent instead of 0.08 M Tris-HCl aq. (pH = 7), and found that fluorescence was successfully recovered (Fig. S1(A)) due to the prevention of autolysis. Based on these results, 0.8 mM HCl aq. effectively maintained the activity of trypsin. However, the fluorescence intensity was still weak compared to that of the batch experiment (Fig. S1(A)), in which autolysis did not need to be considered. Therefore, trypsin solution of a higher concentration (40 μg/mL) was used as a spotting solution to increase the amount of tryps in with maintained enzymatic activity. Using a high-concentration trypsin solution for immobilization resulted in a fluorescence intensity close to that of the batch experiment (Fig. S1(B)).

Based on the above results, we could confirm the successful construction of a single-step trypsin inhibitor assay microdevice. Figure S2 shows the top view of the microchannel. From Fig. S2, the immobilization of two reactive reagents (trypsin and fluorescent substrates) at the two bottom corners of the microchannel was confirmed. Next, the fluorescence images and response curve against measurement time were obtained by introducing the sample solutions containing 10⁻⁸–10⁻² M leupeptin, which has been known as typical inhibitor of trypsin, into the microdevice (Fig. 2). The fluorescence intensity was calculated by subtracting the background fluorescence intensity from the fluorescence intensity of the microchannel. Figure 2(B) shows that the final
fluorescence intensity decreased with the increase in leupeptin concentration, indicating that leupeptin inhibited trypsin, preventing its reaction with the fluorescent substrates. Next, we conducted trypsin inhibitor screening using three kinds of inhibitor candidates as samples. Figure 3 shows the fluorescence intensity 10 min after the introduction of the sample containing leupeptin (using the data shown in Fig. 2(B)), trans-4-(aminomethyl)cyclohexanecarboxylic acid (TAMCHA), or 6-aminohexanoic acid in 0.1 M Tris-HCl buffer (pH = 7). TAMCHA is a reported compound exhibiting relatively low inhibitor activity for trypsin, and 6-aminohexanoic acid is a structurally similar compound to TAMCHA. Unlike those of TAMCHA and 6-aminohexanoic acid, the leupeptin inhibition curve was obtained in a low-concentration region. Therefore, single-step trypsin inhibitor screening using the present microdevice was successfully demonstrated. In the case of TAMCHA and 6-Aminohexanoic acid, maximum fluorescence intensity is slightly lower than that in the case of leupeptin. This might be caused by coexisting high concentrations of TAMCHA and 6-Aminohexanoic acid, that affected the fluorescence intensity of AMC or reduced the activity of the enzyme. In the case of leupeptin, the half-maximal inhibitory concentration (IC$_{50}$) was calculated to be $6.7 \times 10^{-5}$ M. The IC$_{50}$ value reported by Uchiyama et al. and McConnell et al. for trypsin inhibitor assays using leupeptin was $6 \times 10^{-6}$ M and $8.1 \times 10^{-6}$ M, respectively. Therefore, the IC$_{50}$ reported in this study was higher than those reported by the above studies. This difference might be caused by the amount of trypsin. In our method using inkjet printing, the trypsin concentration was higher than the optimized concentration in the batch experiment due to the effect of trypsin autolysis. To avoid autolysis, mixing ionic polymers to restrict enzyme motion, or enzyme-polymer conjugate preparation might be needed. However, a previous report by McConnell et al. described the need of multiple operation procedure. Nevertheless, Uchiyama et al. required a complicated device fabrication for trypsin immobilization on a PDMS substrate using a layer-by-layer deposition. Compared to these methods, present method allowed single-step operation and simpler device fabrication using
inkjet printing. In this study, combination of Boc-Gln-Ala-Arg-MCA and trypsin was evaluated as an example. When different substrates are used, the response will be in different inhibitor concentration ranges, thus comprehensive evaluation can also be possible by the preparation of arrayed device immobilizing various combinations of substrates and enzymes. In addition, since the inexpensive PDMS can be mass-produced and total amount of reagents required to prepare the device is small, disposable and low-cost device fabrication is expected. Recently, smartphone with small objective lens attachment is commercially available, thus, combination with handy-UV lamp would increase the possibility of more convenient use.

Finally, the storage stability of the single-step trypsin inhibitor assay microdevice was investigated by storing it at 15 °C for 1, 2, 4, 7, 10, and 15 days in the shade, and 10^{-2} M leupeptin sample solution in 0.1 M Tris-HCl buffer (pH 7) or 0.1 M Tris-HCl buffer (pH 7) was introduced into the microdevice for evaluation. Figure S3 shows the fluorescence intensities of the microchannels after 10 min. The fluorescence intensity remained unchanged for 10 days. The fluorescence intensity at 15 days slightly decreased, and the error became large when Tris-HCl buffer was introduced. Therefore, the present microdevice can be stored for at least 10 days.
Conclusions

In this study, we present the development of a single-step trypsin inhibitor assay microdevice based on reagent immobilization using inkjet printing. We demonstrated that an acidic solvent was effective for stable protease immobilization. We obtained the inhibition curve by simply introducing sample solutions by capillary action and successfully performed trypsin inhibitor screening. Storage stability was maintained for at least 10 days. In summary, the future application of various enzyme inhibitor assays for drug discovery based on the present immobilization method using enzymes and fluorescent substrates as reactive reagents would be potentially interesting for multiple fields of research.

Acknowledgments

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Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.
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Fig. 1  Principle of a single-step inhibitor assay microdevice based on inkjet printing.
Fig. 2 (A) Fluorescence images of the microchannels 10 min after introducing various leupeptin solution concentrations. (B) Response profile after the introduction of $10^{-8}$–$10^{-2}$ M leupeptin solutions into the microdevice. Error bars show the standard deviation with three replicated measurements (RSD: around 10%).
Fig. 3  Inhibition curve 10 min after introducing the sample containing leupeptin (using the data shown in Fig. 2(B)), *trans*-4-(aminomethyl)cyclohexanecarboxylic acid (TAMCHA), or 6-aminohexanoic acid.
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