Electronic Supplementary Material (ESI) for Lab on a Chip.

Accelerated protein digestion and separation with picoliter volume utilizing nanofluidics

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Electronic Supplementary Information (ESI)

Surface modification:

In the integrated device, PEG-coated, ODS-coated, trypsin-immobilized and bare silica (OH-group) nanochannels were required. Moreover, the distance between the trypsin-immobilized region and the ODS-coated injection chamber should be as short as possible because this distance is the source of the sample injection dead-volume. Moreover, contamination of ODS in the enzyme reactor might cause non-specific adsorption of peptides. Therefore, the challenge was the precise surface patterning of four different materials. To solve these issues, we proposed a surface modification method using valve operation. The procedure of the surface modification is shown in Fig. S1, where the details of the four required steps are shown. All operations for valve closure were conducted with an applied force of F = 0.8 N.

(1) APTES patterning in the enzyme reactor

APTES was partially modified for the reaction field on the upper substrate by vacuum ultraviolet light prior to the bonding process. The two substrates were bonded using a low-temperature bonding method at 110 °C for 3 h.[1]

(2) ODS modification in the separation channel

The surface of the separation channel was modified by introducing a solution including an ODS silanization reagent. Octadecyldimethyl-*N,N*-diethylaminosilane (ODS-DEA) was synthesized from chlorodimethyl-n-octadecylsilane (Alfa Aesar, Ward Hill, MA, USA) and diethylamine (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), as described in a previous study.[2] The synthesized stock of ODS-DEA was diluted in toluene at 20 % (v/v) (ODS-DEA solution). Ethanol, acetone, toluene and ODS-DEA solution were initially

introduced to the microchannels connected to the separation channel. The ODS-DEA solution was than introduced to the separation channel at 50 kPa for 2 h. The temperature was controlled at 70 °C using a Peltier heater. To partially modify ODS, the valve was closed by an actuator as shown in Fig. S1. After modification, the entire channel was washed with toluene at 200 kPa for 30 min. In the ODS modification process, the valve operation was closed.

(3) PEG modification and trypsin immobilization

To reduce the potential for nonspecific protein adsorption onto the nanochannel surfaces, the surfaces were chemically modified with 0.1 wt% PEG in a water/ethanol solution. Previously, PEG modification significantly reduced nonspecific protein adsorption, almost to background level, lower than that of bare silica.[3] For the partial modification of PEG and trypsin, the path of PEG and trypsin solutions was controlled. The microchannels and nanochannels were initially filled with ethanol with the valve open. The PEG solution was then introduced into the reactor channel at an applied pressure of P_1 = 35 kPa. At the same time, ethanol was introduced to the separation channel at an applied pressure of P_3 = 5 kPa for 60 min. Under these conditions, the PEG solution reaching the injection chamber was immediately washed away, thereby avoiding contamination with PEG on the surface of the separation channel. After modification, microchannels and nanochannels (reaction and separation channels) were washed with ethanol for 15 min. The microchannels and nanochannels were then filled with water. As in the PEG modification, reagents for the trypsinogen immobilization process were introduced to the reaction channel and washed away at the injection chamber. Keeping this pressure balance, the trypsin immobilization was performed. Here, trypsin was modified on the APTES-patterning region.

For chemical crosslinking between APTES and trypsin via protein amino groups, a 2.5%

glutaraldehyde solution in 100 mM borate buffer (pH 8.0) was contacted with the APTES coated surface to bind the aldehyde groups. Trypsinogen was coupled to the support by pumping 2.0 mg/mL trypsinogen in 100 mM borate buffer containing 20 mM $CaCl_2$ through the nanochannel at 4 °C for 2 h. Trypsinogen was converted to active trypsin by treatment with 0.01 units enterokinase in 20 mM Tris-HCl (pH 7.4) containing 2 mM $CaCl_2$ and 50 mM NaCl at 23 °C for 2 h. For PEG and trypsin modification, we have selected this applied pressure to achieve the same flow rate and introduced time to compare with a previous report.[3]

Finally, to remove contamination with PEG and trypsin molecules in the ODS-coated separation channel, acetonitrile was introduced to the separation channel and the valve was subsequently closed.

(4) OH-group patterning (hydrophilic)

When the surface of the detection chamber was hydrophobic, the POPS signal was found to be unstable. Although the cause is unknown, it might be attributable to the adsorption of target molecules. Thus, ODS in the detection chamber should be removed. A NaOH solution was used to form the pattern of OH groups. A solution of 0.1 N NaOH was introduced into the detection chamber from microchannel 3. To avoid overflow of the NaOH solution into the separation channel by diffusion, dynamic fluidic control of the NaOH solution was performed. At first, the separation channels were filled with ultra-pure water. Then, the NaOH solution was introduced from microchannel 3 to the detection chamber. The time and flow velocity were controlled so that NaOH reached the detection chamber and did not reach the separation channel. After introduction, NaOH was immediately removed by introducing ultra-pure water from the microchannel from the P_2 side. This process was repeated 30 times over a 10 min period. During this process, the valve was closed.

Evaluation of enzyme reactor performance:

The measurement workflows shown in Fig. 3 were similar to those in the original paper with minor modifications. Briefly, 512 μM BAPNA was introduced to the reaction channel and digested for 0.5, 1.5 and 5 min. The chromatogram is shown in Fig. 7. The peaks were observed at around 60 sec. On the other hand, when the reaction time was 0.5 min, the signal was only slightly observed. Compared to each measurement, the height of the peak was increased as the reaction time increased. As a control sample, a 100 %-digestion sample was also measured. This control sample was digested by 0.01 % mg/mL trypsin for 24 hours in a 2 ml tube at 22 °C. The resulting peak of the 5 min-digestion was comparable to the peak-height of the 100%-digestion sample, indicating that the enzyme reactor achieved almost complete digestion within only 5 min. In conclusion, the enzyme reactor and UV-detection were demonstrated to work in the integrated device.

Cytochrome C properties and amino acid sequence:

The molar absorption coefficients of cytochrome C at 257 nm and 532 nm were experimentally calculated to be 21680 and 12330 cm^{-1} M^{-1}. The amino acid sequence of cytochrome C is shown in Fig. 8. Generally, trypsin can cleave peptide chains at the C-terminal site of lysine (**K**) and arginine (**R**).[4] Considering this, cytochrome C has 21 cleavage sites. Thus, the complete enzyme reaction would generate 22 fragments. Among them, fragments including the amino acids phenylalanine (**F**), tyrosine (**Y**) and tryptophan (**W**) would be detected by UV-POPS. This was because these amino acids are aromatic amino acids and are known to have absorption at 257 nm. The molar absorption coefficient at 257 nm of phenylalanine (**F**), tyrosine (**Y**) and tryptophan (**W**) was 194, 466 and 3224 cm^{-1} M^{-1}, respectively.[5] These values were much higher than other amino acids. If cytochrome C was completely digested into peptides, 8 kinds of fragments including aromatic amino acids would be generated.

Supplementary Figure

Fig. S1

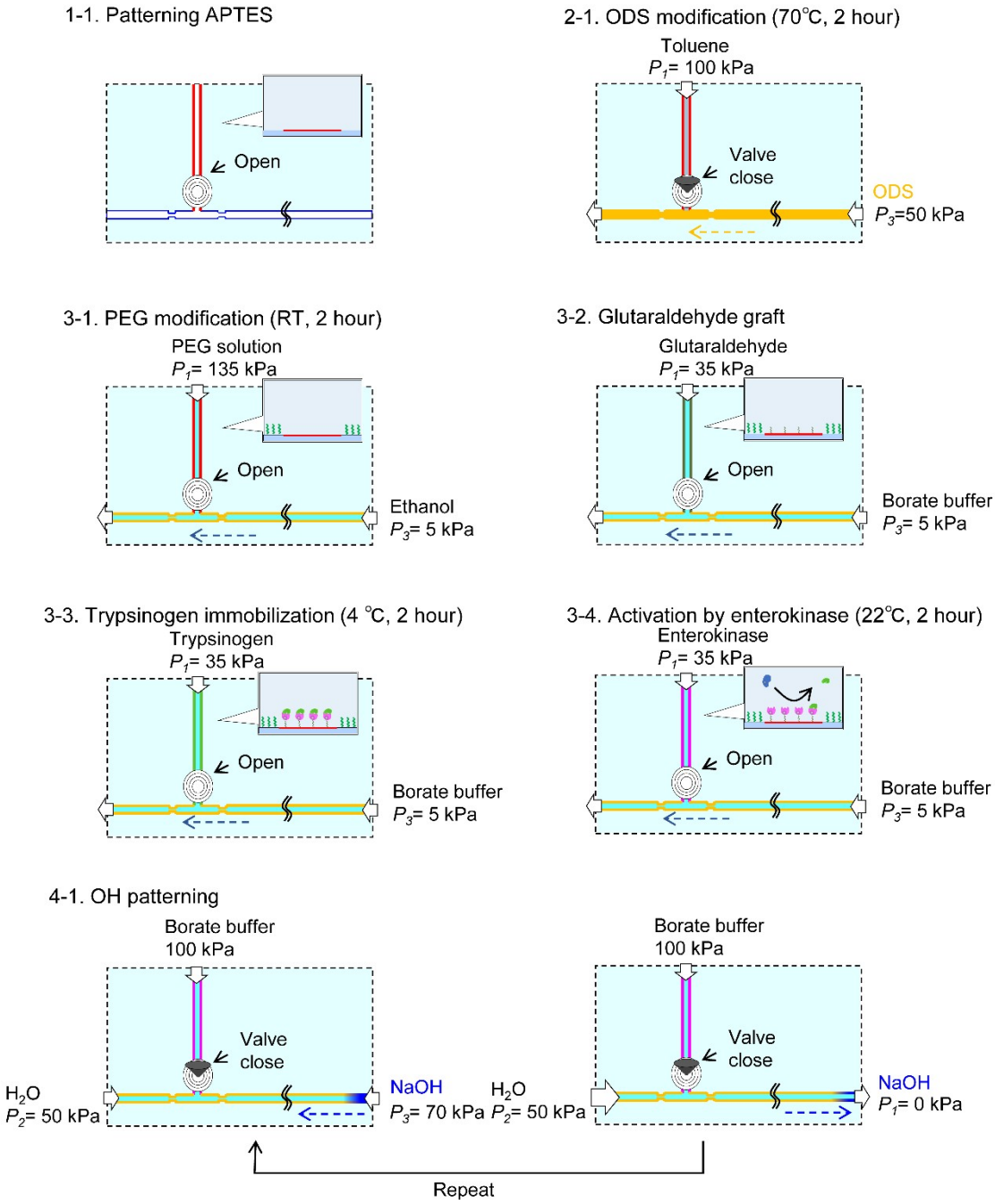

Fig. S1. Layout of surface modifications

Reference.

1. K. Shirai, K. Mawatari and T. Kitamori, *Small*, 2014, **10**, 1514–1522.
2. N. Tanaka, H. Kinoshita, M. Araki and T. Tsuda, *J. Chromatogr. A*, 1985, **332**, 57–69.
3. K. Yamamoto, K. Morikawa, H. Imanaka, K. Imamura and T. Kitamori, *Analyst*, 2020, **145**, 5801–5807.
4. L. Switzar, M. Giera and W. M. A. Niessen, *J. Proteome Res.*, 2013, **12**, 1067–1077.
5. G. d. Fasman, Handbook of Biochemistry, CRC Press, 2018.