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Supporting Information

"One-pot" fabrication of clickable monoliths for enzyme reactors

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Experimental Details

Materials

Tetramethoxysilane (TMOS), (3-iodopropyl)-trimethoxysilane (IPTMS, ≥ 95%), poly(ethylene glycol) (PEG, M_n=10 000), ampliflu red (AR), resorufin, hydrogen peroxide (H_2O_2, 30%), 4-pentynoic acid (PA), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), CuBr, 11-azido-3,6,9-trioxaundecan-1-amine (ATA), propargylamine (PPA), peroxidase from horseradish (HRP), pepsin (from porcine gastric mucosa), and IgG (from human serum) were purchased from Aldrich (Milwaukee, WI). Fused-silica capillaries (365 μm o.d. and 200 μm i.d.) were purchased from Polymicro Technologies (Phoenix, AZ). The water used in all experiments was double-distilled and purified by a Milli-Q system (Millipore Inc., Milford, MA). Other chemical reagents were of analytical grade.

"One-pot" fabrication of azido- and alkynyl-silica hybrid monolithic columns

A fused-silica capillary was pretreated by sequential rinsing with 1.0 M HCl for 12 h, water for 30 min, 1.0 M NaOH for 12 h, and water for another 30 min, and then dried by a stream of nitrogen at room temperature. To prepare completely hydrolyzed silane solution, a mixture of acetic acid (0.02 M, 4.0 mL), methanol (1.0 mL), PEG (10 000 MW, 550 mg), urea (700 mg), TMOS (2.0 mL), and IPTMS (0.5 mL) was stirred at 0 °C for approximately 3.5 h to form a homogeneous solution. Monomers ATA and PPA were separately neutralized with diluted acetic acid (HAc) before use. Typically, 200 μL H_2O and 100 μL HAc were added to 300 μL ATA, while 200 μL H_2O and 350 μL HAc were

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added to 300 μL PPA. For the preparation of the azido-silica hybrid monolithic column (azido-silica monolith), an aliquot of 80 μL neutralized ATA (about 1.25:1 molar ratio of ATA to IPTMS to completely react with iodopropyl moieties) was mixed with 0.5 mL of the hydrolyzed silane solution in a 2-mL centrifuge tube. After vigorous vibration and sonication for about 1 min, this pre-condensation mixture was introduced into the pretreated capillary by a syringe. Both ends of the capillary were then sealed with rubber stoppers, and the capillary was placed in a water bath at 40 °C for 24 h. Finally, the obtained hybrid monolithic columns were sequentially flushed with water (50 μL) and methanol (100 μL) to remove the PEG and other residuals (Scheme 1A). A similar procedure was used to prepare the alkynyl-silica hybrid monolithic column (alkynyl-silica monolith) except that 40 μL of neutralized PPA was used (equivalent to a molar ratio of 1.75:1, PPA to IPTMS) and the incubation temperature was 50 °C (Scheme 1B).

Fabrication of iodopropyl-silica hybrid monolithic column (IP-silica monolith)

One milliliter of the hydrolyzed silane solution (prepared as described previously) was sonicated for 5 min, and introduced manually into the pretreated capillary by a syringe. After both ends of the capillary were sealed with rubber stoppers, the poly-condensation reaction was carried out at 50 °C for 12 h. The obtained IP-silica hybrid monolithic columns were then flushed with water (50 μL) and methanol (100 μL) to remove the PEG and other residuals.

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Evaluation of alkynyl-silica hybrid monolithic column

The chromatographic retention mechanism of the alkynyl-silica monolith was also compared with the IP-silica monolith in capillary liquid chromatography (CLC). The bonding of the polar PPA monomer could decrease the hydrophobic property of IP-silica monolith or even change the hydrophobic surface to hydrophilic. To test the existence of the hydrophilic property of the alkynyl-silica monolith, high percentage acetonitrile solution (95%) was applied as the mobile phase. As shown in Fig. S1, toluene and thiourea were coeluted in the IP-silica monolith which means no hydrophilic retention of polar thiourea. In contrast, on the alkynyl-silica monolith, the stronger hydrophilic retention of thiourea also observed with a partial separation from toluene, which confirmed the successful attachment of the PPA monomer on the monolith (Fig. S1).

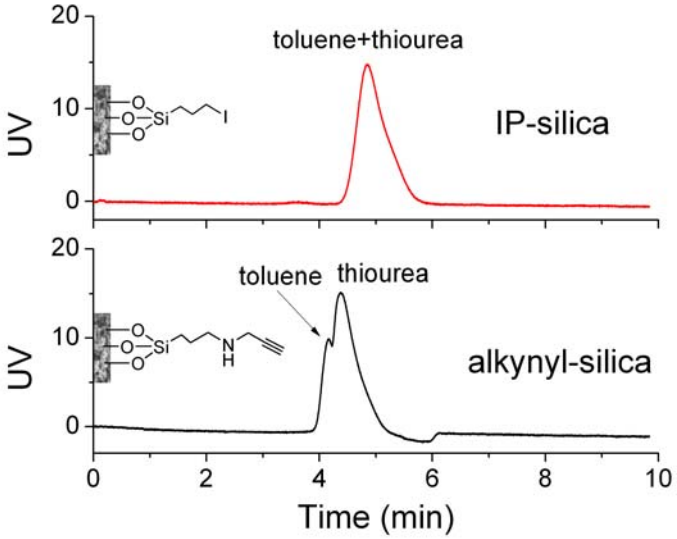

Fig. S1. The comparison of the retention properties among prepared monoliths. Chromatography conditions: mobile phase, 95% ACN with 0.1% formic acid; injection, 1.0 μL in split mode; effective column length, 35 cm; detection wavelength, 214 nm.

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Immobilization of HRP to the azido/alkynyl-silica monoliths via CuAAC

Immobilization procedures were similar to those described in the literature with some modifications.[1, 2] Prior to immobilization on the azido-silica monolith, the enzymes need to be modified with alkynyl groups. PA was chosen as the alkynyl donor and conjugated to HRP. An enzyme solution containing 1.0 mL HRP (2 mg/mL, dissolved in NaH_2PO_4-Na_2HPO_4 buffer of 0.1 M at pH 6.0) and 5 μL PA (20 mg/mL) was prepared at low temperature and incubated at 4 ^oC for 15 min. Subsequently, 1.0 mg EDC was added to the enzyme solution and reacted at 4 ^oC for 3 h. Finally, the solution was transferred to a dialysis bag with the molecular weight cut of 3000 Da, and dialyzed against N_2HPO_4-NH_2PO_4 buffer (0.02 M, pH 6) for 12 h (change the buffer 3 times) to eliminate the unreacted PA and EDC.

In immobilization, 200 μL of 2.0 mg/mL alkynylated HRP (alkynyl-HRP) was mixed with 5 μL $CuSO_4$ (0.1 M) and 5 μL sodium-L-ascorbate (0.2 M) and the mixture was pumped slowly through the azido-silica monolithic column using a syringe at 4 ^oC for 24 h. The column was then flushed with a phosphate buffer (0.1 M Na_2HPO_4-NaH_2PO_4, pH 6, 500 μL) to remove the unbound enzyme and excess reactants.

For the immobilization of HRP onto the alkynyl-silica monolith, HRP was first functionalized with azido groups. The diazo transfer reagent imidazole-1-sulfonyl azide hydrochloride (ISA) was synthesized and conjugated to HRP (azido-HRP) according to a reported method.[3, 4] For the modification of HRP with ISA, 20 μL ISA (30 mg/mL), 5 μL $CuSO_4 \cdot 5H_2O$ (50 mg/mL) and 50 μL K_2CO_3 (20 mg/mL) were mixed with 1.0 mL HRP

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solution (2 mg/mL). Then, the reaction was carried out at 4 ^{o}C for 24. Finally, the mixture was dialyzed as described previously.

Immobilization of the azido-HRP onto the alkynyl-silica monolith was performed in a procedure similar to the immobilization of alkynyl-HRP onto the azido-silica monolith. All the prepared HRP micro reactors, when not in use, were stored at 4 ^{o}C with both capillary ends immersed in water.

Comparison of the enzyme activity of the azido/alkynyl modified HRP

The effect of the modification on HRP enzyme activity was investigated by adding the same amount of enzyme to the same amount of substrate. In detail, 5 μL enzyme solution (azido-HRP, alkynyl-HRP, and intact HRP solution, 1.0 μg/mL each in 0.1 M Na_2HPO_4-NaH_2PO_4 buffer at pH 6), 200 μL AR solution (0.05 mg/mL), and 30 μL H_2O_2 solution (0.001%) were mixed and added to a 96-well plate. The fluorescence intensity was measured by a plate reader (Multimode Detector DTX 880, Beckman Coulter). Fig. S2 shows that the modification of HRP did not significantly decrease its activity.

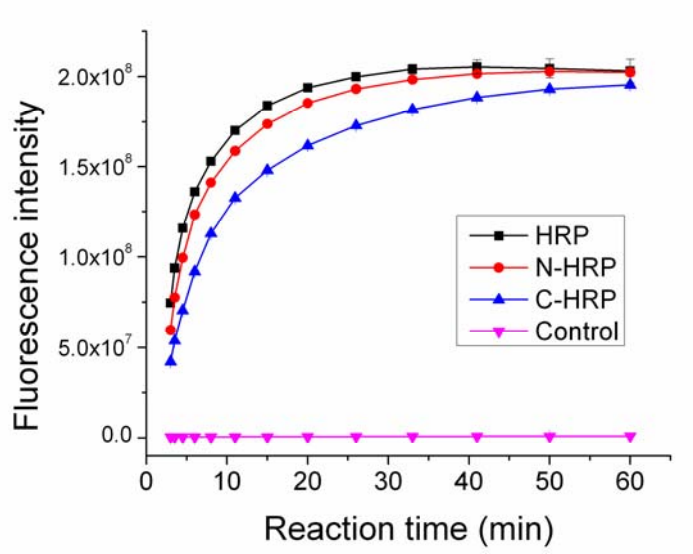

Fig. S2. The comparison of the HRP activity before and after modification with azido- or alkynyl- moieties.

HRP, intact enzyme; N-HRP, azido-HRP; C-HRP, alkynyl-HRP; Control, no enzyme was added.

Evaluation of the hybrid monolithic columns and HRP micro reactors

For evaluation of the retention mechanism of the prepared hybrid monolithic columns (azido-silica, alkynyl-silica, and IP-silica), each of them was connected to a binary micro-HPLC pump (Agilent) in split manner using a Tee-union to reduce the injection volume. The outlet of the column was connected to an open capillary (I.D. 50 μm, O.D. 365 μm) through a short segment of Teflon tubing and the UV detection window was located at 5 cm from the outlet end of the monolithic column.

The performance of HRP micro-reactors was evaluated by connecting them to a micro-HPLC pump (Agilent) equipped with a laser induced fluorescence (LIF) detector (excitation wavelength 543 nm and emission wavelength 580 nm). A mixture of AR and

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H_2O_2 was continuously pumped through the reactor. Breakthrough curves were monitored to judge whether HRP was successfully immobilized,[5] because HRP is able to catalyze oxidation of the weakly fluorescent substrate AR into the highly fluorescent product resorufin by H_2O_2.[6]

Kinetics of the HRP reactor and free HRP at different H_2O_2 concentration

H_2O_2 is the specific substrate of HPR. When the H_2O_2 is combined with HRP, it can be activated and react with many compounds, such as phenol, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), as well as AR in this work. The K_m was measured at different H_2O_2 concentrations while kept AR excess and constant both in solution and on HRP reactor. As shown in Fig. S3, the signal intensity increased accordingly to the H_2O_2 concentration; and the breakthrough time for the curves also were shortened according to the increase of the H_2O_2 concentration. The reaction time for the on the HRP reactor was estimated about 9 s at the flow rate of 20 μL/min. The enzymatic activity of the HRP-reactor was calculated using the frontal chromatography according to the previous reported method.[7] The fitting of the Lineweaver-Burk plots was excellent (R^2=1), and the V_{max} and K_m were calculated as 7.46 mM/min and 1.12 mM for the HRP reactor.

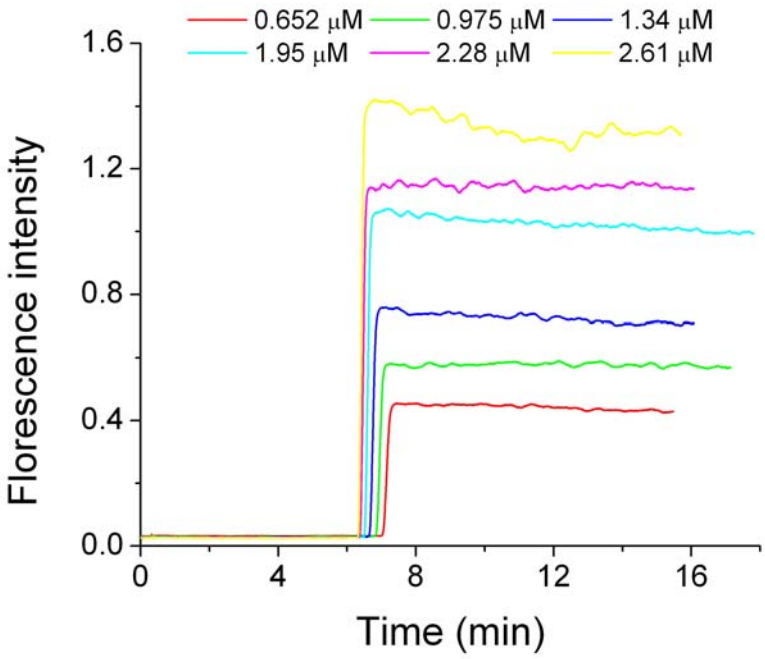

Fig. S3. Frontal chromatograms from HRP reactor at different H_2O_2 concentrations. Conditions: flow rate, 20 μL/min; AR solution, 10 μg/mL in phosphate buffer (pH 6.0). Other conditions were the same as in Figure 4.

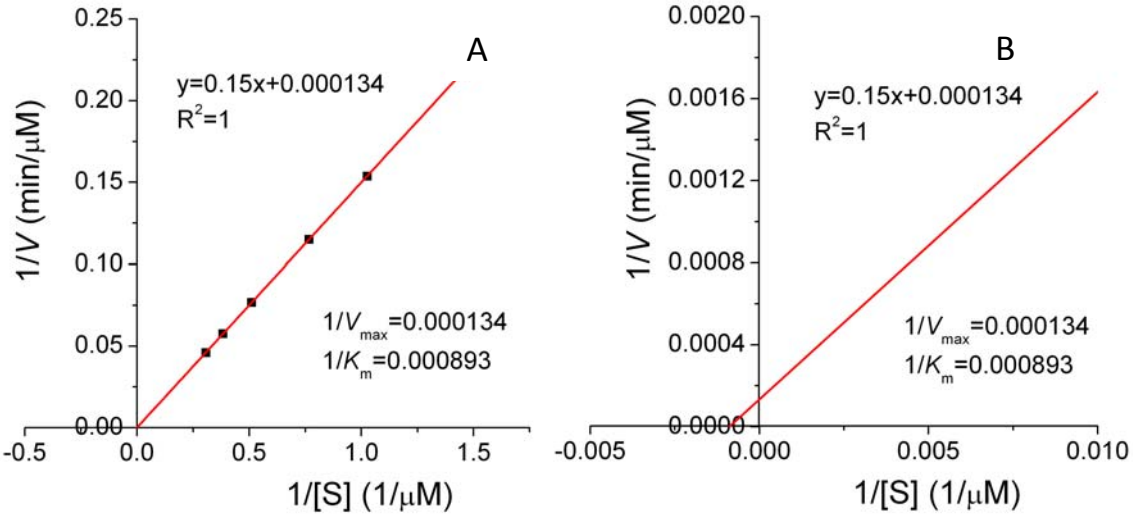

Fig. S4. Lineweaver-Burk plots of the HRP reactor. B is the zoom in of A near the intercept region.

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For the reaction kinetics in solution, different amount of H_2O_2 (2 to 10 μL, 0.0002%) were mixed with 200 μL AR solution (10 μg/mL in 0.1 M phosphate buffer at pH 6.0). After adding of 3 μL C-HRP solution (1 μg/mL), the mixtures were vibrated for several seconds and 100 μL of each were transferred to the 96-well plate. The florescence intensity generated during the reaction was measured by the plate reader and the results were shown in Fig. S5. The initial reaction rate was calculated at the time point of 3 min since it took about 2 min for the mixing and loading. The in solution Lineweaver-Burk plots in Fig. S6 also showed excellent linear fitting (R^2=1) and the V_{max} and K_m in the solution were calculated as 0.35 mM/min and 1.06 mM, respectively. The K_m of the HRP reactor was a little higher compared to that in the solution, however, the V_{max} increased dramatically for about 21 times.

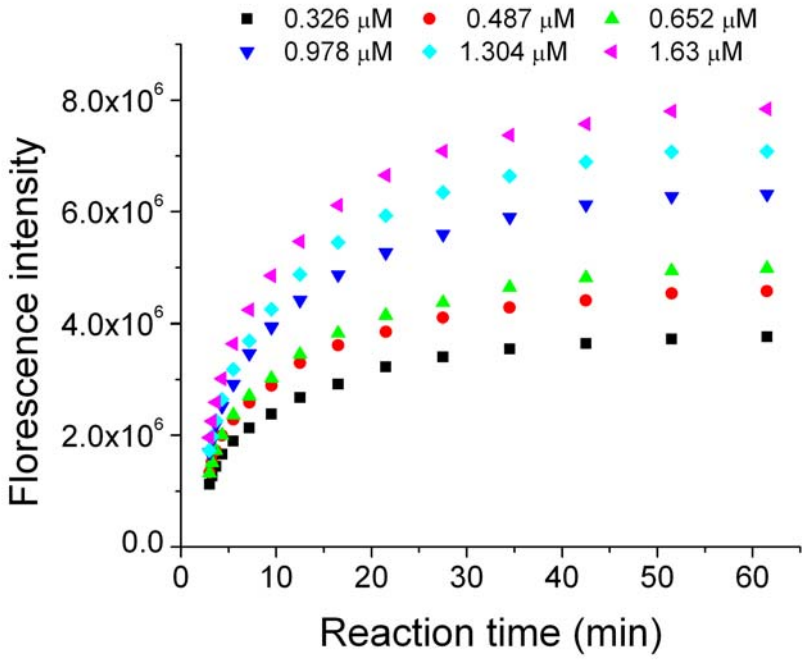

Fig. S5. The in-solution reaction kinetics of C-HRP at different H_2O_2 concentration. Reaction solutions contain 200 μL AR (10 μg/mL, 38.9 μM); 3 μL C-HRP (1.0 μg/mL) and different amount of H_2O_2. Other conditions were the same as in Fig. S2 except the H_2O_2 concentration.

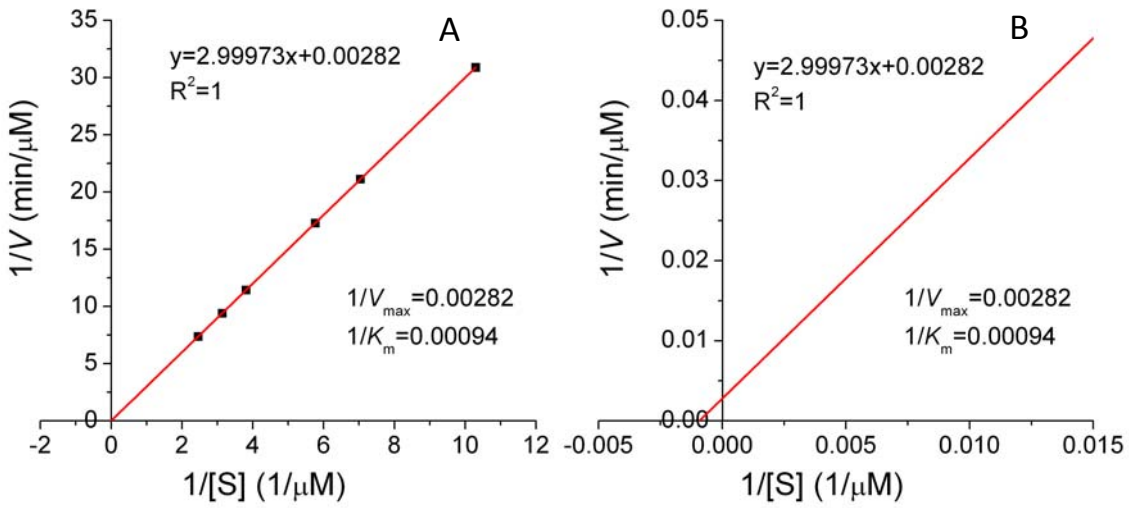

Fig. S6. Lineweaver-Burk plots of the C-HRP in solution reaction. B is the zoom in of A near the intercept region.

Evaluation of HRP reactor prepared from alkynyl-silica monolith

The evaluation of the HRP reactor prepared from the alkynyl-silica monolith was carried out in the same manner. Fig. S7 shows the breakthrough curves observed for this HRP reactor which also confirmed the success of enzyme immobilization. It should be noted that the signal intensity (lower) and the breakthrough time (shorter) was a little different to that of the reactor prepared from the azido-silica monolith. Fig.2 shows the skeleton structure of azido-monolith is thinner than that of alkynyl-monolith, which indicates lower specific surface area of alkynyl-monolith, and leads to the lower capability and shorter break through time. The lower column capacity of alkynyl-monolith could also result a lower amount of alkynyl moieties available on the matrix surface which would further affect the enzyme immobilization amount. One more

reason could be because the accessibility of azido group on azido-monolith for enzyme conjugation is easier than that of alkynyl group on alkynyl-monolith due to the longer linkage arm of ATA. The longer arm is favorable for enzyme immobilization because it could decrease the steric hindrance. The difference in immobilized enzyme amount would affect enzyme reactor activity or signal intensity, and may also affect the retention ability of the reaction product.

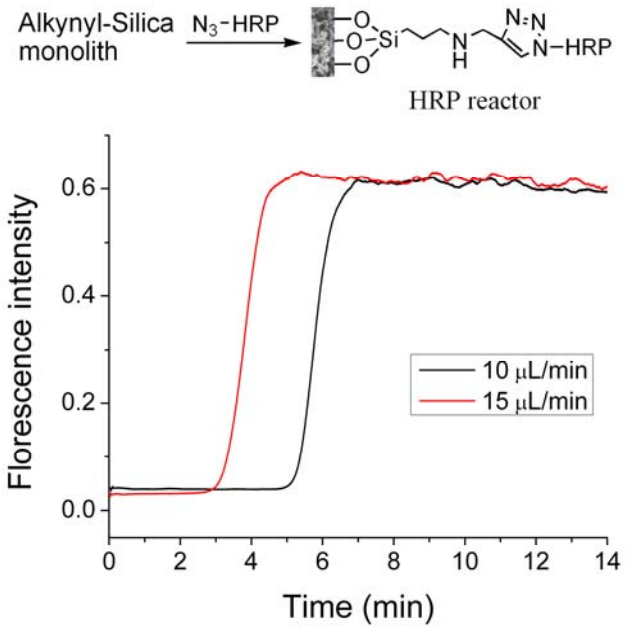

Fig. S7. The frontal chromatogram of HRP reactor prepared from alkynyl-silica monolith. Chromatography conditions: effective column length, 14 cm; mobile phase, 0.1 M phosphate buffer (pH 6) containing 10 μg/mL AR (, 1.0 mL) and 25 μL H_2O_2 (0.0005%).

Preparation and application of pepsin micro-reactor

Pepsin was modified with PA to introduce the alkynyl moiety, and subsequently immobilized onto the azido-silica monolith by a similar procedure to the one described

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for the preparation of HRP reactors. The modification and immobilization were executed at acidic pH (pH 4–4.5) because pepsin can be irreversibly denatured at neutral or alkaline conditions. Pepsin is a nonspecific endopeptidase that can digest IgG into one $F(ab')_2$ fragment which consists of two light chains and two Fd segments (from the two heavy chains), and numerous small peptides from the Fc segment (Fc'). On-column digestion was performed by continuously bumping 0.5 mg/mL IgG through the pepsin reactor at different flow rates (1.0, 2.0, and 5.0 μL/min) and effluent fractions were collected and determined by SDS-PAGE. Control experiments of in-solution digestion were performed at both room temperature and 37 ^{o}C by adding 2.0 μL of 0.4 mg/mL alkynyl-pepsin or intact pepsin to 50 μL of 0.5 mg/mL IgG (mass ratio about 1:30).

SDS-PAGE analysis of the pepsin digestions

Preparation of the 12% resolution gel (20 mL): 40% acrylamide, 3.8 mL; 1.5 M Tris-HCl buffer, pH 8.8, 5.0 mL; 10% SDS, 0.2 mL; H_20 11.0 mL; TEMED, 10 μL; 10% APS, 100 μL. Preparation of stacking gel (10 mL), 40% acrylamide, 1.0 mL; 0.5 M Tris-HCl buffer, pH 6.8, 2.52 mL; 10% SDS, 100 μL; H_2O 6.36 mL; TEMED, 10 μL; 10% APS, 50 μL.

Loading sample preparation: 10 μL of each sample was mixed with 20 μL sample buffer (β-mercaptoethanol : Laemmli buffer = 1:19 v/v) and then incubated at 95 ^{o}C for 5 min. After cooling at room temperature, 6 μL of each denatured sample as well as the marker was loaded onto the gel. Separation voltage was set at 120 V.

When electrophoresis separation was finished, the gel was taken out of the

electrophoresis cartridge and washed with water 3 times. Then the gel was stained in Coomassie brilliant blue (Bio-Rad) for 1 h. After that, the stained gel was washed with water for another 3 times and destained in water on a shaker for 12 h. The image of the gel was taken by Image Quant LAS 4000 (GE Healthcare).

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