Supporting Information for

Ink-jet printing an optimal multi-enzyme system

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Materials

Glucose oxidase (GOx) from *Aspergillus niger* (≥100 units/mg), alcohol dehydrogenase (ADH) from *Saccharomyces cerevisiae* (≥300 units/mg), diaphorase (DP) from *Clostridium kluyveri* (3~20 units/mg), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid)-diammonium salt (ABTS), nicotinamide adenine dinucleotide (NAD^+), 2,6-dichloroindophenol sodium salt hydrate (DCPIP), fluorescein isothiocyanate ,and rhodamine B isothiocyanate were purchased from Sigma-Aldrich. Horseradish peroxidase (HRP) (300 units/mg) was purchased from Nanjing Oddo Foni Biology Technology Ltd. Glucose, Brij®35, bovine serum albumin (BSA), PEG-20000 and NADH were purchased from Beijing BioDee Bio.Tech. Co. Ltd.

The photo paper named 'economy photo paper' is manufactured by SEIKO EPSON Corporation. The multipurpose paper is purchased from DELI Group Co., LTD. The parchment paper (Gateway brand) is produced by Arjowiggins (Quzhou) specialty paper co., LTD., China.

Preparation of the enzyme and substrate inks

For the preparation of HRP or GOx ink, PEG-20000 and *tert*-butanol were added to the enzyme solution (1 mg/mL of protein) in phosphate buffer (pH 7.0, 50 mM) to adjust its viscosity and surface tension. An optimized formulation consists of 140 mg/mL of tert-butanol and 23 mg/mL of PEG-20000, with the surface tension around 36.1 ± 0.2 dyn/cm and viscosity around 3.9 ± 0.2 mPa·s[1]. For the preparation of substrate ink, glucose (100 mM) and ABTS (10 mM) were dissolved in the butanol-PEG-phosphate buffer solution with the same formulation.

For the preparation of ADH or DP ink, ADH or DP was dissolved in phosphate buffer (pH 7.5, 50 mM) at a concentration of 1 mg/mL. To avoid the deactivation of ADH and DP in *tert*-butanol, Brij®35, (1 mg/mL) was used alternatively to reduce the surface tension of the enzyme ink to a value of 44.0 ± 0.3 dyn/cm. In addition, 1 mg/mL of BSA was added to the solution for the stabilization of ADH and DP. 26.7 mg/mL of PEG-20000 was also used to adjust the viscosity to the required value (about 3.1± 0.1 mPa·s). The substrate ink contains 20% (v/v) of ethanol, 30 mM of NAD^+ and 15 mM of DCPIP in the Brij®35-PEG-BSA-phosphate buffer.

Prior to fill in the color cartridges, all of the inks were filtered through a 0.45μm membrane.

[1] The viscosity measuremrnt was carried out on using a. Physica MCR 301 rheometer under a shearing rate at 100 s^{-1} at 25 °C.

Synthesis of FITC-labeled HRP and Rhodamine B-labeled GOx

0.4 mL of fluorescein isothiocyanate in DMSO solution (5 mg/mL) was dropwise added into 4 mL of Na_2CO_3-$NaHCO_3$ buffer (pH=9.0, 50 mM) containing 20 mg of HRP, followed by stirring for 4 h at room temperature. After quenching the reaction with NH_4Cl with a concentration of 50 mM, the solution was dialyzed against phosphate buffer (pH 7.0, 50 mM) for 48 h at 4 ^oC to remove the unreacted fluorescent dye. With the same procedure, Rhodamine B isothiocyanate was used to label GOx.

Enzymatic assays

For HRP, 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid)-diammonium salt (ABTS) was used as the substrate. The enzymatic activity was determined by adding 50 μL of 10 μg /mL HRP solution and 50 μL of 0.3% H_2O_2 to 900 μL of the substrate solution (0.5 mM ABTS in 50 mM phosphate buffer, pH 7.0). The increase of absorbance at 415 nm was recorded for 1 min.

For GOx, 20 μL of 10 μg/mL GOx solution and 80 μL of 10 μg/mL HRP solution were added to 900 μL of the substrate solution (containing 100 mM glucose and 0.5 mM ABTS), followed by measuring the increase of absorbance at 415 nm for 1 min.

For ADH, 10 μL of ethanol was first added to 970 μL of 50 mM phosphate buffer at pH 7.5 containing 0.75 mM NAD^+. Then, 20 μL of 20 μg/mL ADH solution was added to start the assay. The increase of absorbance at 340 nm was determined for 1 min. For DP, 20 μL of 8 mM DCPIP solution was first added into 970 μL of NADH solution (0.3 mM in 50 mM phosphate buffer, pH 7.5). Then, by adding 10 μL of the DP solution (1 mg/mL), the activity of DP was determined by measuring the decrease of the absorbance at 600 nm for 1 min.

All of the above enzymatic assays were taken on the SHIMADZU UV-2450 spectrophotometer at room temperature.

Optimization of enzyme ratios in solution

For GOx and HRP system, 100 μL of mixed enyzmes solution (at different ratios) with total concentration of 10 μg protein /mL was added into 900 μL of phosphate soultion at pH=7.0 (containing 100 mM glucose and 0.5 mM ABTS), the increase of absorbance at 415 nm was determined in 1 min.

For ADH and DP system, 100 μL of mixed enyzmes solution (at different ratios with total concentration of 100 μg protein /mL) was added into 900 μL of phosphate soultion at pH 7.5 (containing 0.3 mM NAD^+ , 0.15 mM DCPIP and 10 μL ethanol), the decrease of absorbance at 600 nm was determined in 1 min.

Laser confocal microscopy

Laser confocal microscopy was carried out on a Zeiss LSM-780 NLO laser scanning confocal system equipped with EC PlnN 10×/0.3 objectives. Samples were pasted on a thin glass slide with drops of deionized water. The excitation wavelengths for FITC-HRP and Rhodamine B-GOx are 488 nm and 590 nm, respectively.

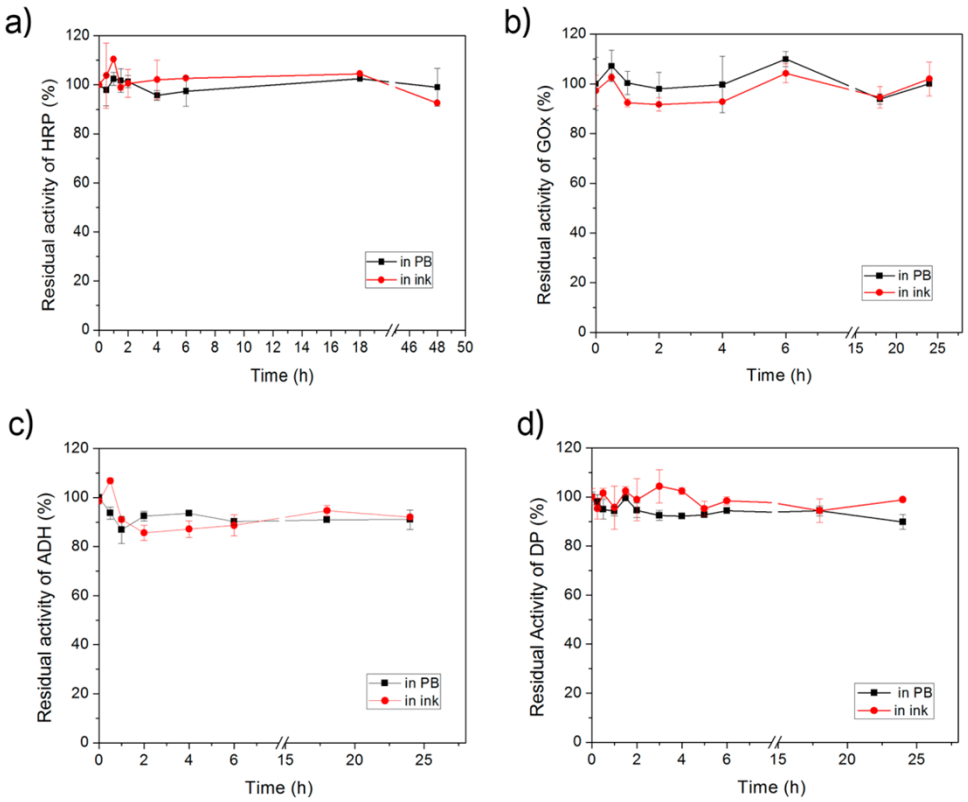

Fig. S1. Stability of enzymes in their ink solutions. (a) HRP and (b) GOx in butanol-PEG-phosphate buffer solution; (c) ADH and (d) DP in Brij®35-PEG-BSA-phosphate buffer solution

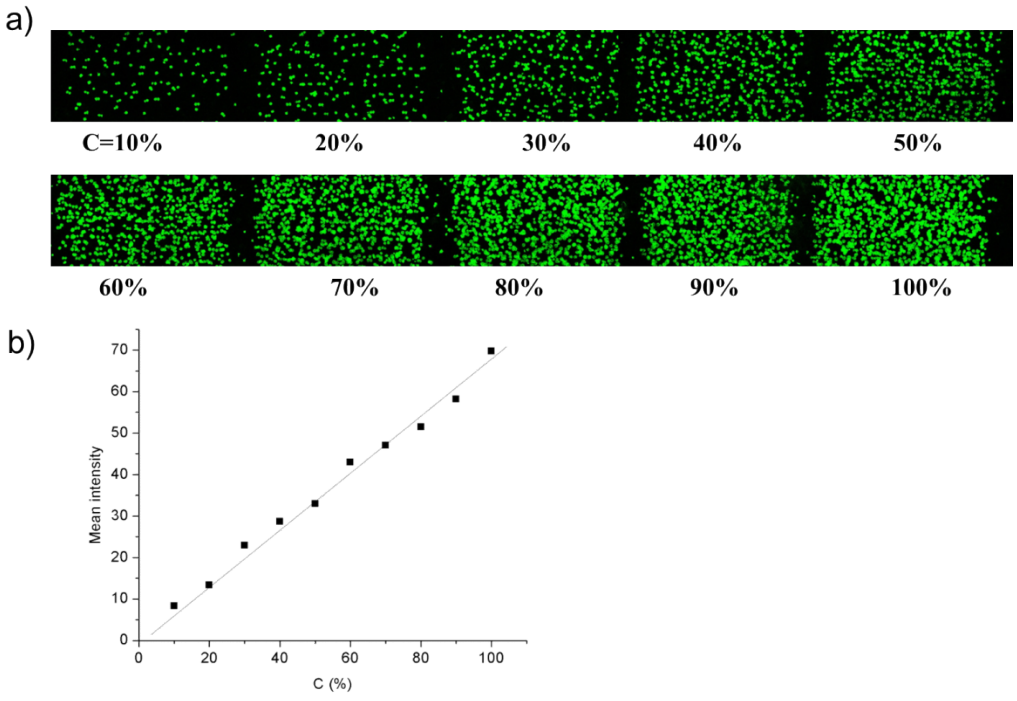

Fig. S2. (a) The laser confocol images and (b) mean intensity of fluorescein-HRP with a gradient of 10% C values.

To determine the drying time, we printed a rectangle (2.5cm *0.5cm) by using the enzyme ink on different papers. The printing process was accomplished in 2~3s. After that, the drying time was recorded when the “water stain” on paper disappeared.

Table S1. The ink drying time on different print substrates.

Print substrate	**Drying time**
Photo paper	<15s
Multipurpose paper	~20s
Parchment paper	~30s
PVC film	70~90s