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Electronic Supporting Information (ESI)

A Logic Circuit upon Angiogenic Response Controlled by Enzyme-linked Iron Oxide Microparticles

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Experimental Section

1. Materials

HUVEC cells (ATCC® PCS-100-010), vascular cell basal medium (ATCC® PCS-100-030), and endothelial cell growth kit (ATCC® PCS-100-041) were obtained from American Type Culture Collection, ATCC® (Manassas, VA, USA). The enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification: GOx from Aspergillus niger, type VII (EC 1.1.3.4); CAT from bovine liver (EC 1.11.1.6). N-hydroxy-succinimide, 98% (NHS), Albumin from bovine serum (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) from Thermo Scientific (Waltham, MA, USA). Dynabeads® MyOne™ Carboxylic acid (Fe_3O_4) and Gibco® Collagen I from rat tail were purchased from Invitrogen (Oslo, Norway). Quick Start™ Bradford Protein Assay kit was purchased from Bio-Rad (Hercules, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) cell growth assay kit was obtained from Millipore (Billerica, MA, USA). Ultrapure water (16.8 MΩ·cm) from MembraPure GmbH (Bodenheim, Germany) was used in making all the buffers; 100 mM MES (pH 6.0), 100 mM Phosphate buffer (pH 7.0), and 10 mM PBS (pH 7.4) were used in this experiment.

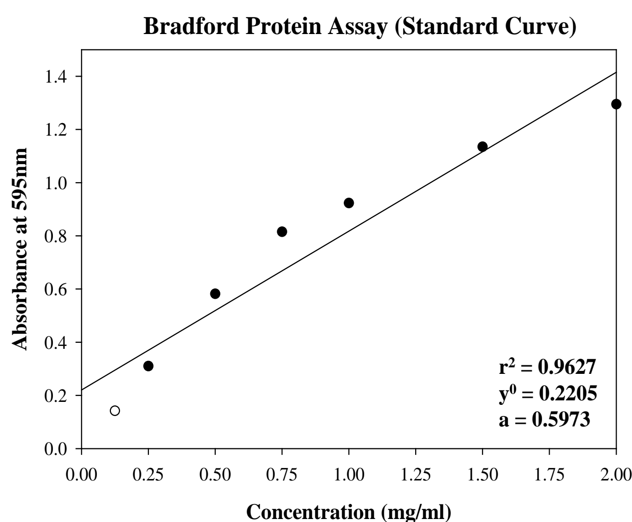
2. Enzyme immobilization onto Fe_3O_4 magnetic particles

Prior to using the MPIOs (Dynabeads®), 3 mg of the particles were washed twice with 1 ml of 100 mM MES, pH 6.0 for 10 min with stirring. EDC solution (50 mg·ml^{-1}) and NHS solution (50 mg·ml^{-1}) dissolved in 100 mM MES, pH 6.0 were prepared, respectively. 75 μl of EDC solution and 75 μl of NHS solution were added to the washed MPIOs, the mixture was mixed well and then incubated with slow tilt rotation at room temperature for 30 min. After incubation, the solution was placed with the magnet for 4 min and the supernatant was discarded. The MPIOs were then washed 4 times using 1 ml of 100 mM MES, pH 6.0. The activated MPIOs were reacted with 1 ml of GOx, CAT, and GOx/CAT mixture in the ratio of 1:1 with the final concentration of 3 mg·ml^{-1} dissolved in 100 mM MES, pH 6.0 with voltaxing, respectively. The enzyme-mixed MPIOs were incubated for 2 h at 4°C with slow tilt rotation. Finally, the MPIOs were washed 4 times using 10 mM PBS, pH 7.4 with the magnet and resuspended with the final concentration of 3 mg·ml^{-1} in 10 mM PBS, pH 7.4. The enzyme coated magnetic particles were stored at 4°C. The concentrations of enzymes onto the MPIOs

were measured by Bradford protein assay kit, respectively.

3. The quantification of GOx loaded on MPIOs using Bradford assay

GOx/MPIOs, CAT/MPIOs, GOx/CAT/MPIOs, and the set of 6 concentrations of BSA for standard from 0.125 to 2 $mg\cdot ml^{-1}$ in 10 mM PBS, pH 7.4, were prepared in 2 ml tubes. 1X dye reagent from 4°C storage was placed under ambient temperature. 1 ml of 1X dye reagent was mixed with 1 ml of each standard or 20 μl of the ENZ/MPIOs with slight voltaxing. The incubation was maintained at room temperature for at least 5 min. After incubation, the absorbance data of the samples was measured at 595 nm using UV-vis spectrophotometer (UV-1800, Shimadzu, Japan) three times, respectively. The concentration of ENZ/MPIOs was determined using the standard curve of BSA protein. The absorbance data at 595 nm of GOx/MPIOs were 0.294, 0.291, and 0.296. In addition, CAT/MPIOs were 0.376, 0.385, and 0.381 as well as GOx/CAT/MPIOs were 0.445, 0.453, and 0.456. From these results, the concentrations of ENZ/MPIOs was calculated using the formula, $y = 0.5973x + 0.2205$.



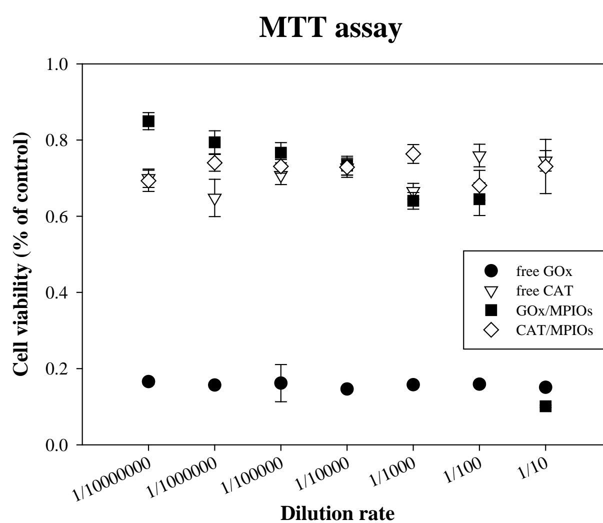
S1. Standard curve of BSA to calculate the concentrations of enzymes onto MPIOs.

4. Cultivation of HUVEC cells and MTT assay for investigation of cell viability

HUVECs were cultured in the vascular cell basal medium with endothelial cell growth kit and 5% (v/v) of antibiotics in humidified incubators at 37°C with 5% CO_2 condition. The medium was changed every 2 to 3 days and HUVECs were split when reached approximately 80% confluence using 0.05% trypsin-EDTA and

incubated for at least 24 h before processing treatment. To carry out the assay for a check of cytotoxicity mediated by complements, such as free GOx, free CAT, GOx/MPIOs, CAT/MPIOs, and GOx/CAT/MPIOs, HUVECs were placed on 10^4 cells per well in 200 μl of complete vascular cell basal medium at 96-well flat-bottomed plates until subconfluent at 37°C with 5% CO_2 incubator. The cells were washed once using steriled PBS buffer and then exposed to serial-diluted components with 100 μl of HUVEC cell pre-cultured media with the complements containing 5% of fetal bovine serum (FBS) on the plates overnight. After the treatment, *in vitro* proliferation assay was performed using MTT assay kit. 10 μl of MTT assay solution was added to each well by tapping gently on the side of the tray for well-mixing. The plates were incubated at 37°C for 4 h. At the end of the incubation, the MTT formazan produced in wells with live cells appeared as dark fuzzy crystals on the bottom of the well. 100 μl of isopropanol with 0.04 N HCl were added to each well and mixed carefully with repeated pipetting. Finally, the absorbance of the plates was measured at 570 nm as well as 630nm for reference using SpectraMax M5 (Molecualr Device, USA).

From the comparison of MTT assay with free enzymes and immobilized enzymes onto MPIOs, free GOx was highly toxic to the HUVECs. However, the cell viability was not affected by free CAT, CAT/MPIOs and even GOx/MPIOs below certain concentration. Generally, free GOx is known as toxic to the cells. We assume that the cytotoxicity of free GOx may be resulted from the internalization of GOx into HUVECs. The immobilization of GOx onto the large-sized MPIOs may prevent the internalization of GOx.



S2. The MTT assay for cell viability with free enzymes and immobilized enzymes onto MPIOs.

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5. Spheroid formation of HUVEC cells

The petri dish with confluent HUVEC monolayer (passage 6) was washed using 1X Hank's Balanced Salt Solution (HBSS) and then HBSS was removed by aspiration. The HUVEC cells were trypsinized using 1 ml of 1X Trypsin-EDTA solution (diluted in 10 mM of autoclaved PBS) for 5 min at 37°C and the reaction was stopped by pipetting 5 ml of PBS on the cells. After pipetting, the cells were put into a reaction tube and centrifuged at 3000 rpm for 10 min. The supernatant was discarded and the cells were resuspended in a culture media with 20% (vol/vol) of methyl cellulose solution (MCS). 10 μl of the cell suspension for each spheroid was divided into non-adherent petri dishes using a 8-channel pipette. The plates were turned upside down and incubated in a humidified atmosphere at 37°C overnight.

6. 3D gel preparation of HUVEC and Angiogenesis control using enzyme-coated MPIOs

600 μl of 10X medium 199 (M199), 120 μl of NaOH and 4.8 ml of collagen were mixed well using gentle up and down of pipetting on ice and 120 μl of $NaHCO_3$ was added to achieve a neutral pH following the addition of 5% FBS solution. 60 μl of the collagen was placed into a 96-well plate immediately and then incubated until a firm gel was formed in 37°C over 15 min. The HUVEC cell spheroids was carefully collected and centrifuged at 500 rpm for 5 min. The supernatant was discarded and the spheroids were resuspended to the mixture of collagen (the same process as above) on ice. The collagen including spheroids were additionally put into the 96-well plate beforehand and then incubated again at 37°C for 30 min. After incubation, the presence of the spheroids inside each well was checked by a microscope (IX51, Olympus, Japan). 80 μl of the media solution with VEGF protein (20 ng·ml^{-1}) and enzyme coated MPIOs of 1000 times the diluted concentration were placed into a 96-well plate and then finally incubated at 37°C overnight. The presence or absence of the sprouts by angiogenesis was measured using a microscope and the image was imported by a digital camera (DP71, Olympus, Japan).

7. Fractal dimension analysis for angiogenesis

Fractal dimension was calculated by a box-counting method using fractal dimension analysis program (FrakOut! v 1.0.0). The overlapped boxes against HUVEC cell microscope images were counted and then fractal dimension was automatically calculated. The different values of fractal dimensions for the samples were

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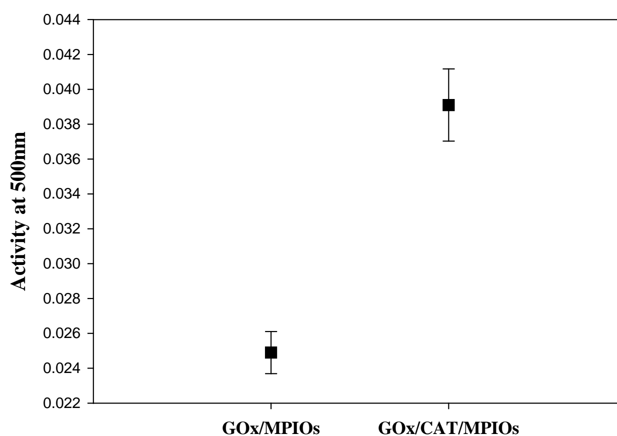
represented as a bar graph.

8. Enzyme activity measurement of GOx/MPIOs and GOx/CAT/MPIOs

980 µl of the reaction buffer including 10 µl of 3.79 mg·ml^{-1} horseradish peroxidase solution in 100 mM phosphate buffer (pH 7.0) and 10 µl of 3 mg·ml^{-1} GOx/MPIOs or GOx/CAT/MPIOs were prepared in a cuvette with 555 mM ß-D-(+)-glucose solution and 0.21 mM *o*-dianisidine solution by 1:4.8 volume ratio. The change of absorbance at 500 nm was measured by UV-vis spectrophotometer (Shimadzu UV-1800PC, Kyoto, Japan). The activity of enzyme was calculated by the slope of time-dependent absorbance change.



S3. Enzyme activity measurement of enzyme immobilized MPIOs.

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