Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Development of temperature dependent oxygen releasable nanofilm by modulating oxidation state of myoglobin

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1. Materials and Methods

Materials

Myoglobin (Mb) from equine heart and Dulbecco's phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (Missouri, USA). Sulfuric acid, hydrogen peroxide, sodium hydroxide and sodium chloride were purchased from FUJIFILM Wako Pure Chemical Corporation (Kyoto, Japan). L (+) - Ascorbic acid (AA), Dulbecco's modified Eagle medium (DMEM, 08458–16) and cell count reagent SF were purchased from Nacalai tesque (Kyoto, Japan). Antibiotics (50 U/mL penicillin and 50 μg/ml streptomycin) was purchased from Thermo Fisher Scientific (MA, USA). Hydrochloric acid was purchased from KISHIDA CHEMICAL Co., Ltd (Osaka, Japan). Fetal bovine serum (FBS) was purchased from MP Biomedical LLC (California, USA). Extracellular O_2 consumption assay (fluorescent oxygen probe) was purchased from Abcam (Cambridge, UK). Normal human dermal fibroblast (NHDF) was purchased from LONZA (Basel, Switzerland). Collagen Type 1 was kindly provided by Nippon ham (Osaka, Japan). We use Milli-Q grade deionized water (Merck Millipore, Burlington, MA, USA). All regents were used without any purification.

Method

Reduction of metMb by AA

MetMb solution was prepared by dissolving Mb powder in 1xPBS solution at a concentration of 200 μM. AA was added to Mb solution at a concentration of 2 mM and NaOH solution was also added to adjust pH at 7. Mb solution containing AA was kept at 37 °C and UV-vis absorption spectra was measured for 48 hours using UV-vis spectrophotometer (V-670M, JASCO, Tokyo, Japan). UV-vis absorption spectrum of Mb solution without AA was also measured. AA solution was prepared at a concentration of 2 mM and NaOH solution was also added to adjust pH at 7. AA solution was kept at 37 °C and UV-vis absorption spectra were measured for 48 hours by diluting AA solution to 100 μM with 1xPBS.

Oxygen absorption to Mb during the course of the reduction of metMb

Oxygen partial pressure of 200 μM Mb solution containing 2 mM AA in 1xPBS was measured every 10 minutes for 120 minutes at 37 °C using oxygen partial pressure measurement device (Bio Research Center Co., Ltd, Tokyo, Japan) and Thermo Alumi Bath (IWAKI, Shizuoka, Osaka) soon after the addition of AA to Mb solution. Additionally, UV-vis absorption spectra changes of 200 μM Mb solution containing 2 mM AA in 1xPBS were measured every 10 minutes for 120 minutes at 37 °C soon after the addition of AA to Mb solution.

UV-vis absorption spectra changes of Mb by additional addition of AA

100 μM Mb solution containing 1 mM AA at pH7 was prepared as mentioned above and incubated at 37 °C. Every 24 hours, 500 μM AA was added to Mb solution. UV-vis absorption spectra were

measured at 2, 24, 26, 48 and 50 hours after the addition of AA to Mb solution.

Degradation of AA

AA solution was prepared by dissolving AA powder in 1xPBS solution at a concentration of 2 mM and NaOH solution was added to adjust pH at 7. Prepared AA solutions were incubated at 20 °C for 4 hours and then incubated at 20, 37 or 45 °C. After 4, 24, 48 hours incubation at each temperature, UV-vis absorption spectra of AA solutions were measured by diluting AA solution to 100 μM with 1xPBS.

Oxidation of oxyMb at various temperature

200 μM Mb solution containing 2 mM AA at pH7 was prepared as mentioned above. Prepared Mb solutions were incubated at 20 °C for 4 hours and then incubated at 20, 37 and 45 °C. After 4, 24, 48 hours incubation at each temperature, UV-vis absorption spectra of Mb solutions were measured.

Oxygen release during the course of the autoxidation of oxyMb

200 μM Mb solution containing 2 mM AA at pH7 was prepared as mentioned above. Prepared Mb solution was incubated at 20 °C for 4 hours and then incubated at 20, 37 and 45 °C. After 4, 24, 48 hours incubation at each temperature, dissolved oxygen amount of 4 mL Mb solution was measured using Multiparameter DO Meter (HANNA, Woonsocket, USA). For the control sample, 1xPBS solution was incubated at 20, 37 and 45 °C for 48 hours and dissolved oxygen amount was measured at 4, 24 and 48 hour using Multiparameter DO Meter.

Long-term storage of oxyMb and the on-off release of oxygen with temperature changes

200 μM Mb solution containing 2 mM AA at pH7 was prepared as mentioned above. Prepared Mb solution was stored at 4 °C for 16 days. Dissolved oxygen amount was measured at 2 day and 16 day by DO Meter and the pictures of Mb solution were also taken. After 16 days storage at 4 °C, Mb solution was incubated at 45 °C for 8 hours. Dissolved oxygen amount was measured at 4 hour and 8 hour and pictures of the Mb solution were also taken. Finally, Mb solution was stored at 4 °C again for 6 days. Dissolved oxygen amount was measured at 17 day (from prepared day) and 23 day and pictures of the Mb solution were also taken. The ratio of dissolved oxygen increase was calculated as below.

[(DO amount of later) – (DO amount of former)] x100 / (DO amount of former)

$(Mb/Col)_5$ multilayered nanofilm preparation and characterization by quartz crystal microbalance

The quantitative analysis of LbL assembly was performed using a quartz crystal microbalance (QCM). At first, the QCM chip (An AT-cut quartz crystal (9 mm diameter) with a parent frequency

of 9 MHz, USI, Fukuoka, Japan) was treated by Piranha solution (H_2SO_4/30% H_2O_2 aqueous solution = 3:1 in volume) twice. Following the cleaning step, the QCM chip was alternately immersed into Mb solution (10 mg/mL, pH 5.0, 1xPBS) and collagen type I solution (1.0 mg/mL, pH 7.4, 1xPBS) for 15 min at 20 °C. Between each step, the QCM chip was rinsed with 1/50xPBS solution, and then dried under N2 gas. The alternate steps were repeated until 5 bilayers. For each step, frequency shift (ΔF) was recorded using frequency counter (53131 A, USI, Fukuoka, Japan), and the amount (Δm) of deposition was obtained by calculating according to the Sauerbrey equation: $-\Delta F$ (Hz) = 1.15 Δm (ng).

$(Mb/Col)_5$ multilayered nanofilm preparation and characterization on quartz cell by UV-vis spectrophotometer

Before LBL assembly, 10 mg/mL Mb solution in 1xPBS at pH 5.0 was mixed with 20 equivalents concentration of AA and kept at 20 °C for 3 hours. The quantitative analysis of LbL assembly was performed on quartz cell using UV-vis spectrophotometer. At first, the quartz cell (TOSOH, Tokyo, Japan) was treated by Piranha solution twice. Following the cleaning step, prepared Mb solution and collagen type I solution (1.0 mg/mL, pH 7.4, 1xPBS) was alternately immersed into quartz cell for 15 min at 20 °C. Between each step, the quartz cell was washed with milli Q, and then dried under N2 gas. The alternate steps were repeated until 5 bilayers. For each step, UV-vis absorption spectrum was measured with 1xPBS.

Autoxidation of oxyMb at (Mb_6/Col_5) multilayered nanofilm with temperature change

(Mb_6/Col_5) film was prepared on quartz cell as mentioned above. After preparation, UV-vis absorption spectra changes were measured with 1xPBS for every 1 °C from 20 to 45 °C.

Oxygen release from (Mb_6/Col_5) multilayered nanofilm

(Mb_6/Col_5) film was prepared on micro fluorescent quartz cuvette (GL science, Tokyo, Japan) as mentioned above and then 350 μL PBS solution containing fluorescent oxygen probe was added. The concentration of oxygen probe was followed by the protocol of the company. To prevent the oxygen diffusion, the solution was covered with accessory mineral oil. After 30 min incubation at 37 °C in the dark condition, fluorescent spectrum was measured using spectrofluorometer (FP-8500, JASCO, Tokyo, Japan). As a control, fluorescent spectrum of oxygen probe in non-coated quartz cuvette was also measured in the same procedure.

Cell culture on (Mb_{11}/Col_{10}) multilayered nanofilm culture dish under hypoxia condition

Before LbL assembly, 10 mg/mL Mb solution in 1xPBS at pH 5.0 was mixed with 20 equivalents concentration of AA and kept at 20 °C for 3 hours. Prepared Mb solution and collagen type I solution

(1.0 mg/mL, pH 7.4, 1xPBS) was alternately immersed into 35 mm tissue culture dish (IWAKI, Tokyo, Japan) for 15 min at room temperature. Between each step, the culture dish was washed with milli Q. The alternate steps were repeated until 21 steps.

$1x10^5$ cells of NHDF were seeded on culture dish coated with oxyMb/Col I LbL nanofilms and non-coated culture dish and 2 mL of DMEM containing 1% antibiotics was added. Culture dishes were incubated in BBL GasPak system (Becton, Dickinson and Company, New Jersey, USA) with two anaeropack O_2 absorber (MITSUBISHI GAS CHEMICAL COMPANY, INC., Tokyo, Japan) at 37 °C. WST assay reagent was prepared by mixing DMEM containing 10 % FBS and 1 % antibiotics and cell count reagent in the ratio of 9:1. After 24 hours incubation, DMEM was removed and 1 ml of WST assay reagent was added. Then, the cells were incubated under normal oxygen condition for 1 hour at 37 °C. 100 µL WST solution of each culture dish were collected in 96-well plate transwell (3470, Corning, New York, USA) and absorbance at 450 nm was measured using SYNERGY/HTX multi-mode reader (BioTek Instruments, Inc., Winooski, USA). As a control sample, $1x10^5$ cells of NHDF were seeded on non-coated culture dish and incubated under normal oxygen condition for 24 hours at 37 °C. WST assay was also conducted in the same procedure. 100 % cell viability was defined from the absorbance of control cells. Phase contrast images of each sample was taken after 24 h incubation using EVOS™ XL Core Imaging System (Thermo Fisher Scientific, MA, USA).

Statistical Analysis

All data are expressed as the means ± standard deviation (SD), unless otherwise specified. The values represent the means ± SD from independent experiments. Student's t-test was run to assess the variance. Test gave p values considered significant if $*p < 0.05$ and $**p < 0.01$.

2. Supplementary Figures

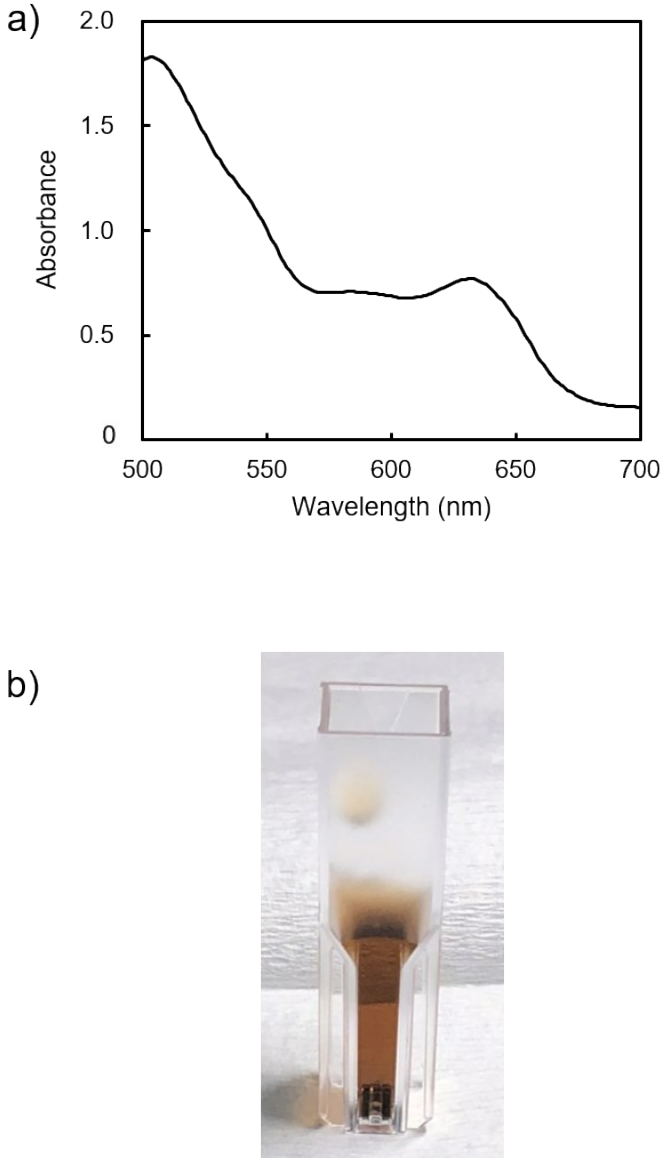


Figure S1. (a) UV-vis absorption spectrum of 200 μM metMb in PBS. (b) The picture of 200 μM metMb in PBS.

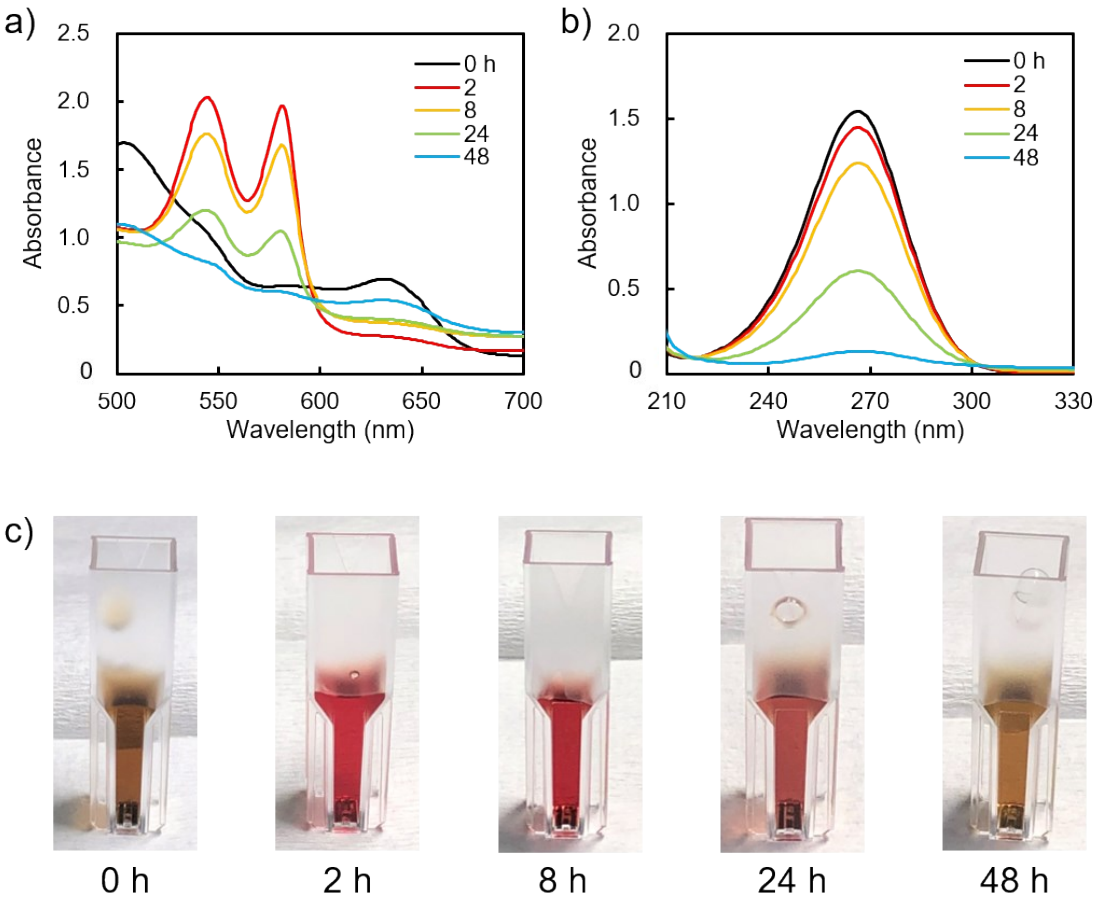
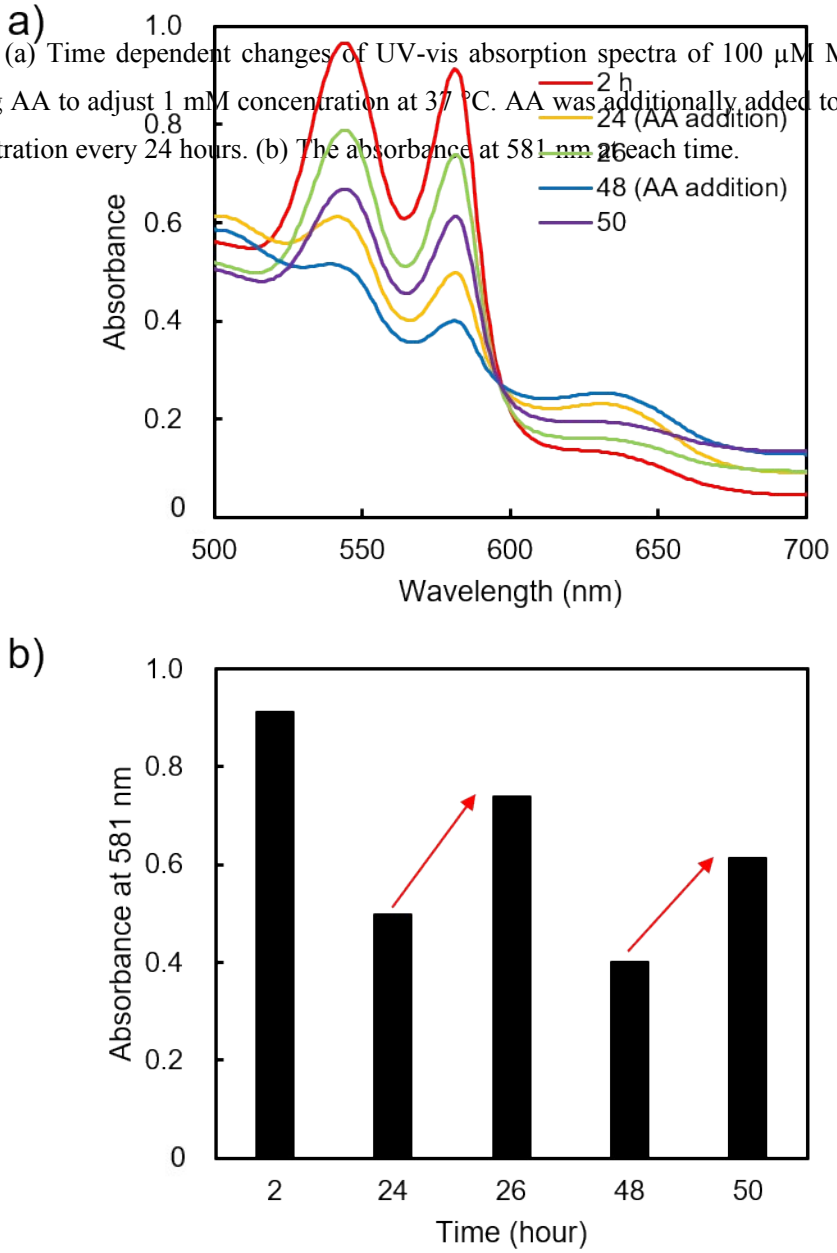


Figure S2. Time dependent changes of UV-vis absorption spectra of (a) 200 μM Mb in PBS after adding AA to adjust 2 mM concentration at 37 °C and (b) 100 μM AA at 37 °C. AA concentration (b) was diluted from 2 mM to avoid absorbance saturation of UV-vis analysis. (c) The pictures of time dependent changes of 200 μM Mb in PBS after adding AA to adjust 2 mM concentration at 37 °C.

Figure S3. (a) Time dependent changes of UV-vis absorption spectra of 100 μM Mb solution after adding AA to adjust 1 mM concentration at 37 °C. AA was additionally added to adjust 500 μM concentration every 24 hours. (b) The absorbance at 581 nm at each time.



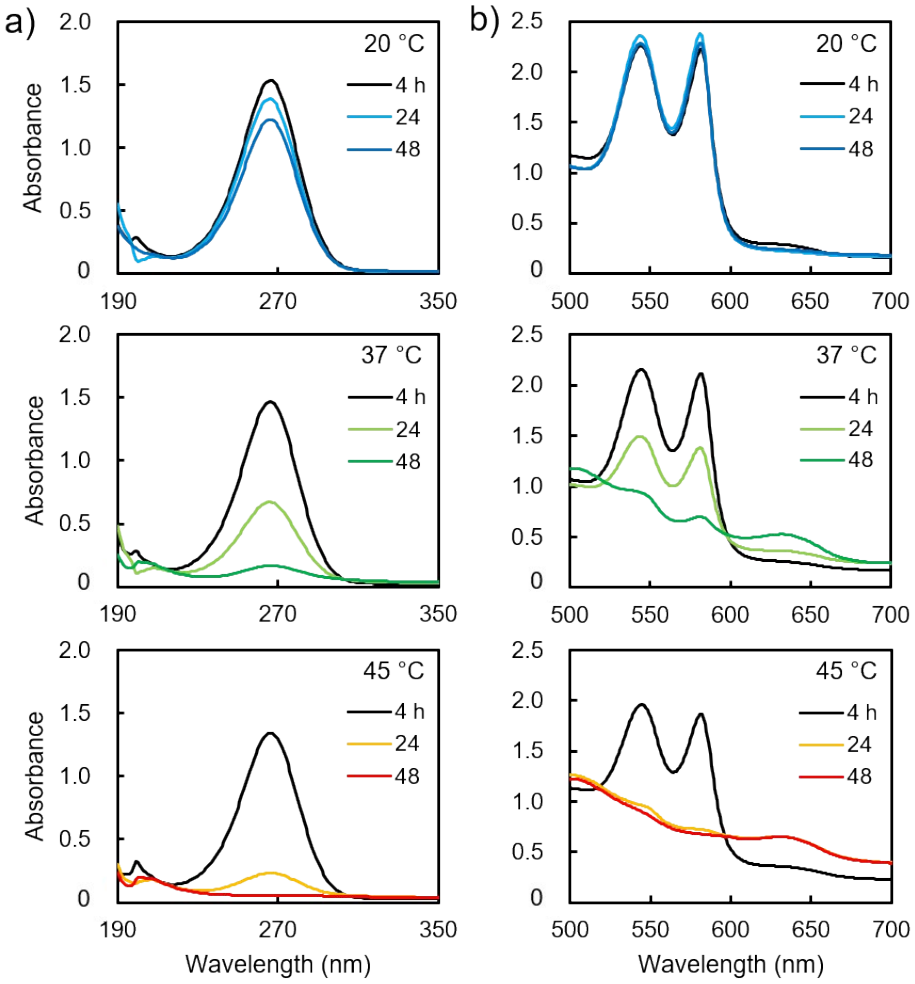


Figure S4. (a) Time dependent changes of UV-vis spectra of (a) 100 μM AA in PBS and (b) 200 μM Mb containing 2 mM AA in PBS at 20, 37 and 45 °C, respectively. AA concentration (a) was diluted from 2 mM to avoid absorbance saturation of UV-vis analysis.

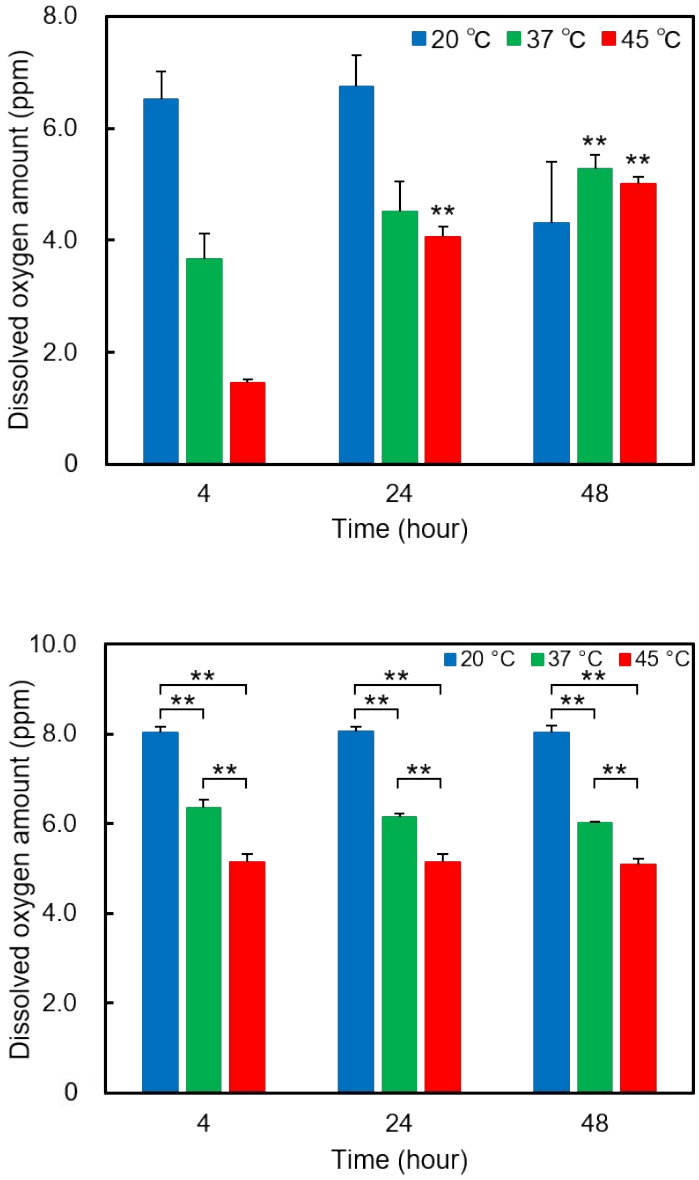


Figure S5. (a) Time dependent changes of the dissolved oxygen amount in (a) 200 μM Mb solution containing 2 mM AA and (b) PBS as a control at 20, 37 and 45 °C, respectively (n=4). Statistical comparisons between the asterisk and others at the same (a) temperature and (b) time were analyzed using two-tailed Student *t*-tests. A *p*-value **< 0.01 was considered to be statistically significant.

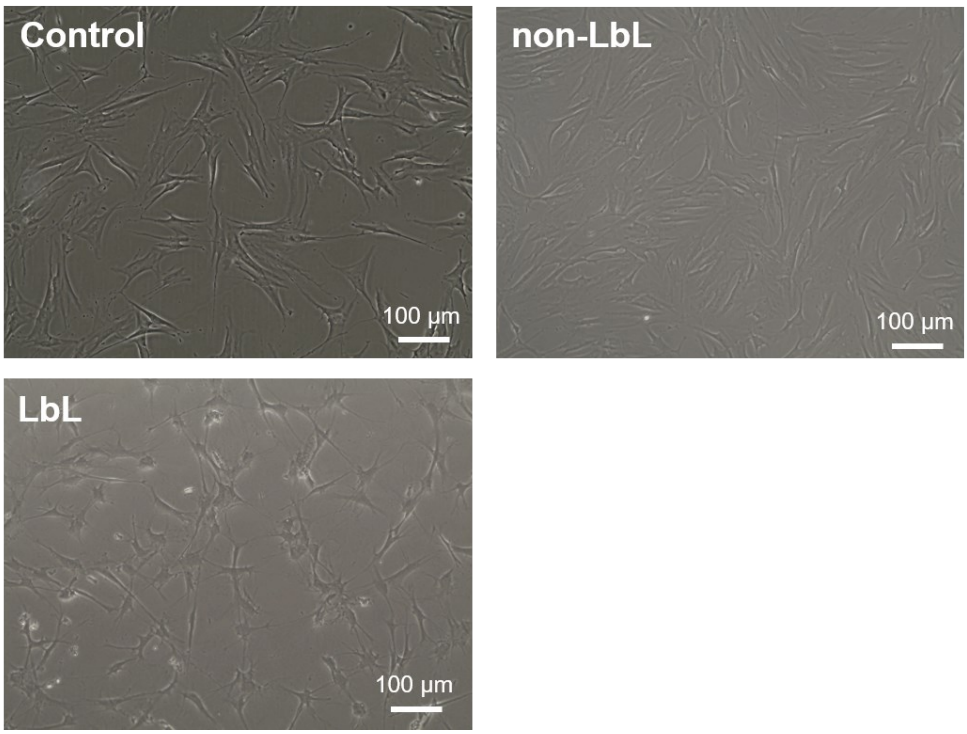


Figure S6. Phase contrast images of NHDF cultured on non-LbL dish under normal oxygen condition (Control), non-LbL dish and LbL nanofilms coating dish (LbL) under hypoxia condition.