Liposome-mediated amplified detection of cell-secreted matrix metalloproteinase-9

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Electronic Supplementary Information
**Experimental Details:**

**Synthesis of lipo-peptide:** The procedure for synthesis, purification and characterization of lipo-peptide have been reported previously.\(^1\)

**Preparation of hydrating HRP buffer solution and eluant buffer:** A stock solution of HRP (100 mM) was prepared in 5 mL of 25 mM HEPES buffer containing 100 mM NaCl, 10 mM CaCl\(_2\) and 10 µM ZnCl\(_2\). The pH of the solution was adjusted to 8.0 and the osmolarity was measured using a standard osmometer. A 2000 mL stock solution of 25 mM HEPES buffer (pH = 8.0) was also prepared. This buffer initially contained 100 mM NaCl, 10 mM CaCl\(_2\) and 10 µM ZnCl\(_2\). Further required amount of NaCl was added to adjust the osmolarity of the stock buffer to that of the HRP solution. This buffer was used as the eluant.

**Preparation of liposomes:** Stock solution of POPC (2 mg/mL, commercially available from Avanti Polar Lipids, Alabaster, AL) was prepared in chloroform. Stock solution of lipo-peptide was obtained by dissolving pure peptide in a mixture of methanol and chloroform (1:4). The final concentration of this solution was 1 mg/mL. A lipid mixture was obtained by adding 1 mL of POPC solution, 2.6 mL of peptide solution and 1.5 mL chloroform in a 10 mL round bottom flask. The mixture was vortexed for 5 min and then subjected to rotary evaporation at 40 °C to remove the organic solvents. A thin film adhering to the sides of the round bottom flask was formed after rotary evaporation. The thin lipid film was dried overnight under high vacuum to ensure complete removal of any residual solvent. The dried lipid film was then hydrated with 1.2 mL HRP solution in 25 mL HEPES buffer at pH = 8.0 for 1 h at 50 °C by fast rotation in a rotary evaporator. The hydrated solution was subjected to repeated freeze and thaw cycles (20 times) to increase encapsulation efficiency. The liposome solution was subsequently stored at 4 °C for 2 h to ensure proper folding of the lipo-peptides into triple helices. The multilamellar vesicles were broken to small unilamellar vesicles by probe sonication for 1 h at 45 °C followed by extrusion (10 times) through polycarbonate membrane filters (100 nm pore size). Liposomes encapsulating HRP was separated from unencapsulated protein by gel filtration using columns packed with hydrated Sephacryl G-200 (GE Healthcare Biosciences). Before actual loading, the Sephacryl G-200 column was equilibrated with 500 mL eluant buffer. The osmolarity of the eluant buffer was adjusted and made equal to that of the hydrating buffer to prevent rupture of the liposomes due to osmotic shock. After loading of the liposomal solution, the buffer eluted from the column was collected in small fractions (1 mL) in test tubes. The phosphate content in each test tube was analyzed following the protocol outlined in phospholipid C test kit (Wako Laboratories). Only the test tubes which tested positive for POPC content were considered for further experiments.
Detection of recombinant human MMP-9: MMP-9 was detected by measuring the intensity of absorbance at 410 nm of the brown solution formed as a result of HRP mediated oxidation of its substrate o-phenylenediamine (OPD). In a typical experiment, desired amount of MMP-9 was added in to a solution containing 10 µL liposomes, 10 µL freshly prepared OPD, 5 µL 3% H₂O₂ and eluant buffer in a total volume of 200 µL. The experiments were performed in 96-well absorbance clear microplates and were set up in triplicates for each set of data. The data was collected in a spectrophotometer (Spectramax, Molecular Devices) and analyzed with Origin 8.0 (OriginLab Corporation).

Determination of the linear dynamic range: In order to determine the linear dynamic range of the detection system we noted that the duration of the linear kinetic phases in Figures 1 and 2 depend on the added enzyme concentration. To prepare a calibration curve for our amplified detection system for MMP-9, the absorbance changes after 25 minutes were plotted as a function of added enzyme concentration (Figure S1). We observed that the absorbance change at 410 nm increased linearly with increasing concentration of MMP-9, up to 200 nM.

![Absorbance Change (410 nm) vs [MMP-9] (nM)](image)

**Equation:** y = mx
slope: 0.0089
R²: 0.997

**Figure S1.** The absorbance change (410 nm) 25 minutes after the addition of MMP-9 is shown as a function of the enzyme concentration (blue circles). The error bars for the measurements are also indicated. The fitted line for the data points using the equation y = mx is also depicted (red line). The slope of the fitted straight line is indicated on the plot.
Detection of cell-secreted MMP-9: All cells were obtained from American Type Culture Collection (Manassas, VA, USA). MCF7 (human breast adenocarcinoma) and HT-29 (human colorectal adenocarcinoma) cell lines were cultured in Eagle’s Minimum Essential medium, and McCoy’s 5a medium, respectively at 37 °C in humidified atmosphere containing 5% CO₂. All media were supplemented with 5% penicillin and streptomycin solution and 10% fetal bovine serum. When the cells achieved 70% of confluency, the old culture media were replaced with fresh culture media without phenol red. The cells were further incubated for 24 h, collected, and centrifuged. The supernatants were collected for further experiments. The experimental set up was very similar as that of recombinant MMP-9 detection. Instead of adding desired volume of recombinant MMP-9, 50 µL of the conditioned media from cultured cell lines or 50 µL of the cell culture media was added to a total volume of 200 µL solution containing liposomes, OPD, 3% H₂O₂ and eluant buffer.

ELISA protocol for determining MMP-9 concentration in conditioned media: To measure the concentration of MMP-9 present in the conditioned media from MCF7 and HT-29 cells, an ELISA was performed using a Human MMP-9 ELISA kit obtained from RayBiotech, Inc. (Catalog No: ELH-MMP9-001), and manufacturer’s instructions were followed. After obtaining the calibration curve using the provided samples of recombinant human MMP-9, the conditioned media (50 µL) from the MCF7 and HT-29 cells were added. The absorbance of this solution was recorded (450 nm) and the concentration of MMP-9 in the conditioned media was estimated from the calibration curve.

References