

Supporting Information

Platelet-derived Nanovesicles for Hemostasis without Release of Pro-inflammatory Cytokines

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Experimental details

1. Materials

Citrate-phosphate-dextrose buffer, bovine thrombin, prostaglandin E₁ (PGE₁), and calcium chloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). A micro-bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). CellTracker Red CMTPX was purchased from Invitrogen (Carlsbad, CA, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, penicillin/streptomycin (P/S), and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Quanti-iT™ PicoGreen® dsDNA assay kit and GelCode™ Blue Staining reagent was obtained from ThermoFisher Scientific (Waltham, MA, USA). Mouse tumor necrosis factor (TNF)- α and interleukin (IL)-6 enzyme-linked immunosorbent assay (ELISA) kit were purchased from BD Bioscience (Franklin Lakes, NJ, USA). Mouse IL-1 β ELISA kit was purchase from R&D systems (Minneapolis, MN, USA). Institute of Cancer Research (ICR) mice (6-8 weeks of age, female) were purchased from Orient Bio Inc. (Seongnam, Kyunggi-Do, Korea).

2. Isolation of platelets and preparation of platelet derived vesicles

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Konkuk University and approved by the Animal Ethics Committee of Konkuk University. Mice platelets were isolated as previously described.¹ Platelets from whole blood were isolated by gradient centrifugation. A 1-mL volume whole mouse blood was mixed with 0.8 mL of citrate-phosphate-dextrose buffer and centrifuged at 100 \times g for 20 min at 25 °C. Afterwards, the supernatant was centrifuged at 800 \times g for 20 min at 25 °C. To obtain the platelet derived vesicles, platelets were suspended in a hypotonic buffer (20 mM Tris-HCl, 10 mM KCl, and 2 mM MgCl₂) in 0.5 mL and sonicated using a Digital Sonifier® 450 (Branson, Danbury, CT, USA). Size of platelet derived vesicles was reduced by optimization of sonication intensity. The condition of sonication was 30% power for 40 sec (20 sec pulse on and 10 sec pulse off). The entire solution was centrifuged at 16,000 \times g for 20 min at 4 °C. The pellet was discarded and supernatant was stored at 4 °C until use.

3. Characterization of platelet vesicles

The amount of protein in platelets and platelet vesicles was determined using the BCA protein assay kit according to the manufacturer's protocol. Prepared platelet vesicles (100 μ g) were dispersed in 0.1 mL deionized water at a final concentration of 1 mg/mL and measured using dynamic light scattering (DLS) using a Nano-S device (Malvern Instrument Ltd, Malvern, UK). To test the stability of platelet vesicles, 71.2, 105, and 253.6 μ g of the preparation was dispersed in deionized water at concentrations of 712, 1050, and 2536.1 μ g/mL and incubated at 4 °C for 0, 24, 48, 72, and 96 h, respectively. Then, each preparation was examined by DLS. The same concentrations of platelet vesicles were dissolved in 10% FBS in hypotonic buffer or 10% FBS and 2 μ M PGE₁ in hypotonic buffer and incubated at 4 °C. The size of the particles in solution was measured by DLS. For transmission electron microscopy (TEM) analysis, 10 μ g of platelet vesicles dispersed in 0.1 mL of deionized water was loaded onto a 300-mesh copper grid and air dried. After washing the grid three times with deionized water, the sample was stained with 2% uranyl acetate solution. Excess uranyl acetate was removed by washing and the grids were observed using a Bio-TEM instrument (Tecnai G² Spirit Twin; FEO; Hillsboro, OR, USA). For quantitative analysis of nucleic acids in platelet lysate and

platelet vesicle, PicoGreen assays were performed according to the manufacturer's protocol. Platelet lysate and platelet vesicle samples were dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and mixed with Quanti-it™ PicoGreen® dsDNA reagent at a sample/reagent volume ratio of 1 for 5 min at room temperature. After incubation, the fluorescence intensities of the solutions were measured using a fluorospectrophotometer at an excitation and emission wavelength of 480 and 520 nm, respectively. Protein migration profiles of platelet lysate and platelet vesicle were measured using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The 10 µg of platelet lysate and platelet vesicle were loaded onto wells of 10% SDS-PAGE and electrophoresis was performed for 45 min. Protein in gel was stained with coomassie blue overnight and de-stained with deionized water overnight.

4. Platelet and platelet vesicle aggregation measurements

Platelets (protein amount of 71.2 µg) were dispersed in Dulbecco's phosphate buffered saline (PBS) with or without 0.3 U/mL thrombin overnight and then centrifuged at $800 \times g$ at 4 °C for 10 min and fixed with 3.7% formaldehyde solution in PBS for 1 h. Primary dehydration of the fixed platelets was performed using 70% v/v ethanol (EtOH) in deionized water for 15 mins. They were then transferred to secondary dehydration solution (90% v/v EtOH in deionized water) for 15 min then to absolute ethanol for 15 mins for total platelet dehydration. Finally, the samples were loaded onto a silicon wafer and desiccated overnight in a desiccator. Scanning electron microscopy (SEM) was done using a S-4800 microscope (Hitachi, Tokyo, Japan). Platelet vesicles (71.2, 105 µg) dispersed in 0.1 mL PBS solution were mixed with or without 5 mM of calcium chloride and with 0.3, 3, 30 U/mL thrombin and incubated at 37 °C in a CO₂ incubator. The size of samples was measured by DLS at 0, 3, 24, and 48 h. Platelet vesicles (71.2 µg) dispersed in 0.1 mL PBS solution were stained with CMTPX 3 µM for 40 min at 37 °C and mixed with calcium chloride and thrombin at a concentrations of 5 mM and 0.3 U/mL, respectively. The platelet vesicles were dispensed in wells of a 96-well plate and incubated for 0, 3, 24, and 48 h at 37 °C in a CO₂ incubator. Platelet vesicles (71.2, 253.6 µg) were stained with CMTPX 3 µM for 40 min at 37 °C and mixed with thrombin at a concentration of 0.3 U/mL. The platelet vesicles were dispensed in wells of a 96-well plate and incubated for 0 and 4 days at 37 °C in a CO₂ incubator, as described above. The platelet vesicles were observed by confocal microscopy using an LSM 710 device (Carl Zeiss, Oberkochen, Germany).

5. Cytokine release study

Platelets dispersed in DPBS were counted after staining with 0.4% trypan blue in PBS. The platelets were centrifuged at $800 \times g$ for 10 min and 5×10^8 cells were dispersed in in RPMI containing 10% FBS.² The platelet vesicles that were not purified or by centrifugation were prepared from the 5×10^8 platelets. The platelets and platelet vesicles (6 mg) were dispensed in wells of a 24-well plate and incubated at 37 °C in a CO₂ incubator with or without 0.5 U/mL thrombin. After 4 days, the samples were centrifuged at $16,000 \times g$ for 20 min at 4 °C and TNF-α, IL-6, and IL-1β were determined using ELISA kits according to the manufacturer's protocol.

6. Bleeding time

The tail transection and bleeding time measurement assay is a simple and well-established model to determine efficacy of hemostatic therapies.³ Mice were injected via the tail vein with buffer solution (4.9 mM Tris-HCl, 10 mM KCl, 1.5 mM MgCl₂, 137 mM NaCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) in PBS solution (control) or the solution containing platelets or platelet vesicles (30 mg/kg in 0.1 mL). In these studies, 7-10 mice were used per group. Thirty minutes after injection, the end of each tail (2 mm) was completely transected. The injured tail was immersed in 14 mL of 37 °C PBS solution and the time required for the first arrest of bleeding was recorded.⁴

7. Statistical analysis

Data in this study represent mean values of independent measurements. Error bars mean standard deviations of each experiment. Statistical analysis was performed with one-way ANOVA combined with Tukey's test. Statistical significance was assigned for $P < 0.05$ (95% confidence level).

References

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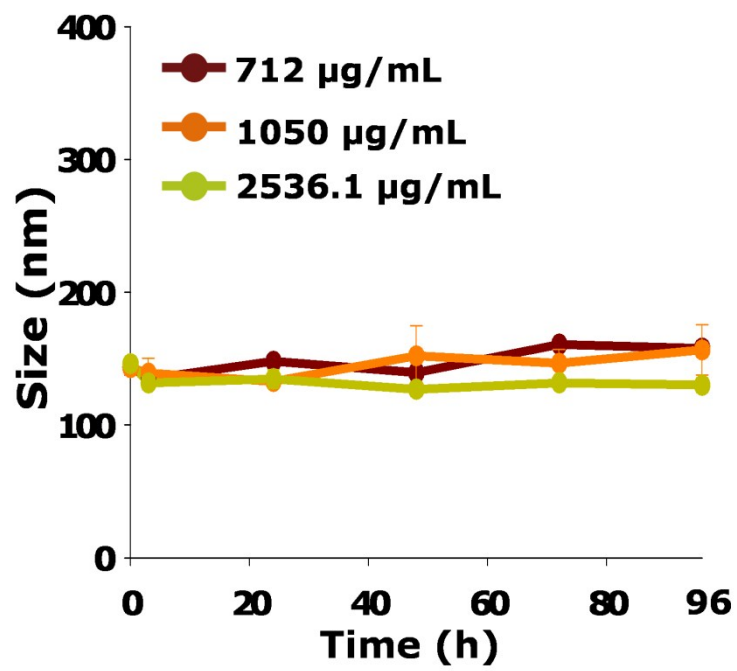


Figure S1. (a) Size stability of platelet vesicles at platelet vesicle protein concentrations of 712, 1050, and 2536.1 µg/mL.

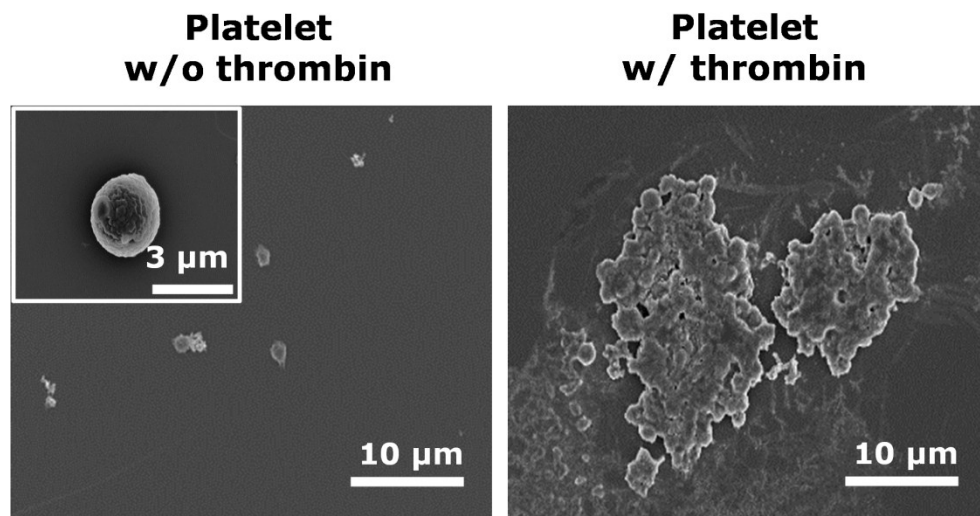


Fig S2. Representative scanning electron microscopy images of platelets incubated with or without thrombin 0.3 U/mL.

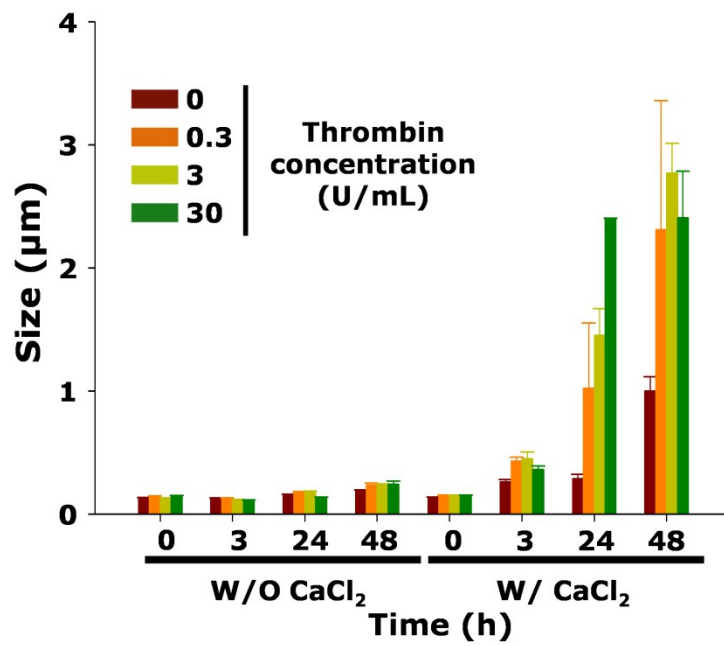


Fig S3. Size change of platelet vesicle aggregates (1050 µg/mL) incubated at 37 °C for 48 h in the presence of four different thrombin concentrations and 5 mM calcium chloride.