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Activated Platelet-Derived Vesicles for Efficient Hemostatic Activity

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In this study, activated platelet-derived vesicles (Act-VEs) are developed as a novel hemostatic biomaterial. Spherical Act-VEs (114.40 ± 11.69 nm in size) with surface charges of −24.73 ± 1.32 mV are successfully prepared from thrombin-activated murine platelets with high surface expression of active glycoprotein IIb/IIIa (GP IIb/IIIa, also known as αIIbβ3) and P-selectin. Although nanosized vesicles from resting platelets (VEs) and Act-VEs showed similar sizes and surface charges, Act-VEs formed much larger aggregates in the presence of thrombin and $CaCl_2$, compared to VEs. After incubation with fibrinogen, Act-VEs formed much denser fibrin networks compared to platelets or VEs, probably due to active αIIbβ3 on the surfaces of the Act-VEs. After intravenous injection of the Act-VEs, tail bleeding time and the blood loss are greatly reduced by Act-VEs in vivo. In addition, Act-VEs showed approximately sevenfold lower release of pro-inflammatory interleukin-1β (IL-1β) during incubation for 4 days, compared to platelets. Taken together, the formulated Act-VEs can serve as a promising hemostatic biomaterial for the efficient formation of fibrin clots without releasing pro-inflammatory cytokine.

Platelets, which originate from megakaryocytes, play an important role in hemostasis after vascular injury and medical surgery.[1–3] After vascular tissue get injured, transmembrane proteins on the surfaces of resting platelets such as glycoprotein IIb/IIIa (GP IIb/IIIa, also known as αIIbβ3), glycoprotein Ib-V-IX complex, glycoprotein VI can bind to exposed sub-endothelial proteins such as von Willebrand factor (vWF) and collagen, which leads to morphological and biochemical changes of platelets resulting in platelets activation.[4–7] Then, activated platelets release agonists such as thromboxane, thrombin, adenosine diphosphate and intracellular calcium ions (Ca^{2+}), which facilitate the formation of fully activated αIIbβ3 and biochemical aggregates for hemostasis.[4,8,9] It is well known that the active form of αIIbβ3 binds to fibrinogen with a much stronger affinity than its resting form, which is one of the crucial factors for the formation of dense fibrin clots.[8]

Based on clot formation mechanisms, diverse hemostatic dressings have been designed over the past several years.[10,11] Inorganic hemostats such as zeolite-based "QuikClot" and clay-based hemostatic agents "WoundStat" have been developed for preferred clot formation. However, there are some challenges in these inorganic hemostats, for example heat-induced tissue damage and occlusive thrombus that reduces blood flow in the vessels.[11–13] To mimic platelets, several peptides and antibodies that bind to vWF, collagen, αIIbβ3, and fibrin have been conjugated onto liposomes and polymeric particles.[14–17] However, there are still challenging limitations in platelet-mimicking particles, including complicated conjugation processes and easy clearance by immune systems.[18–21] To overcome these limitations, we recently proposed the fabrication of platelet-derived nanosized vesicles (VEs) as a novel hemostatic biomaterial.[22] VEs with whole membrane proteins of platelets and high surface to volume ratios have allowed efficient interaction with fibrinogen and other endogenous platelets for successful hemostatic activity in vitro and in vivo. Furthermore, our VEs released negligible amounts of pro-inflammatory cytokines during aggregation compared to platelets, which might result in less activation of macrophages.[22]

In this study, to improve the hemostatic activity of VEs, vesicles were prepared not from resting platelets but from activated platelets. It was hypothesized that activated platelet-derived vesicles (Act-VEs) with an active form of αIIbβ3 could provide high affinity to fibrinogen and fibrin for successful and prompt formation of fibrin clots and hemostasis. The physicochemical properties of Act-VEs (e.g., hydrodynamic sizes, surface charge, and morphology) were examined by dynamic light scattering (DLS) and transmission electron microscopy (TEM) analysis. The clot-forming properties of VEs and Act-VEs were comparatively determined by size measurement. The degree of fibrin clotting by platelets, VEs, and Act-VEs was determined by scanning electron microscopy (SEM) and confocal microscopy. After tail-vein injection of platelets, VEs, and Act-VEs, the bleeding time from the injured vein were analyzed in vivo by using the filter paper method. The amount of pro-inflammatory cytokine interleukin-1β (IL-1β) was quantitatively measured by enzyme-linked immunosorbent assay after incubating platelets and Act-VEs for 4 days.

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According to a previous study, the activation of platelets with thrombin resulted in a release of calcium signaling, transformation of resting αIIbβ3 to active αIIbβ3, and high expression of P-selectin on membrane surface.[4] It should be noted that the binding affinity of active αIIbβ3 to fibrinogen and fibrin is much higher than that of resting αIIbβ3.[8] In this study, it was hypothesized that VEs with active αIIbβ3 would have favorable characteristics for hemostasis in vivo. After activation of platelets, the level of protein expressions including active αIIbβ3 and P-selectin were examined by fluorescence-activated cell sorting analysis, as shown in Figure S1a, Supporting Information. Noticeable shift of cell population was observed for activated platelets after staining with both fluorescein (FITC) labeled P-selectin antibody at the left panel and pycoerythrin labeled active αIIbβ3 antibody at the right panel of Figure S1a, Supporting Information, which indicates that activated platelets expressed much higher amount of P-selectin and active αIIbβ3 on their membrane than resting platelets. The relative percentage of cell population within a gate region for control, resting platelets, and activated platelets were 0.1%, 47.0%, and 70.0% for P-selectin expression and 0.0%, 60.5%, and 89.6% for αIIbβ3 expression, respectively. According to previous study, active αIIbβ3 on activated platelets has much higher affinity to fibrinogen than inactive form, which could allow prompt coagulation as efficient bridges.[6,8]

Then, two types of platelet-derived vesicles, VEs and Act-VEs, were prepared from resting platelets and activated platelets, respectively, as shown in **Figure 1**a. Through the sonication of resting or activated platelets in hypotonic solution, VEs and Act-VEs were prepared, according to our previous study.[22] As shown in Figure 1b, Act-VE could form large density fibrin clot in the presence of fibrinogen, $CaCl_2$, and thrombin. After intravenous injection of platelets, VEs, and Act-VEs, the amount of bleeding from the injured vessel was monitored to evaluate the hemostatic effects of each biomaterial (Figure 1c) in vivo.

The characteristics of the formulated VEs, including size, morphology, surface charges, and protein patterns, were investigated as shown in **Figure 2**. The hydrodynamic diameter of Act-VEs was 114.40 ± 11.69 nm (Figure 2a), which is similar to that of VEs according to our previous study.[22] The surface charges of VEs and Act-VEs were −27.33 ± 2.28 and −24.73 ± 1.32 mV, respectively (Figure 2b). The morphology of Act-VEs was visualized by TEM (Figure 2c; Figure S1b, Supporting Information). Spherical and cup-shaped Act-VEs were successfully observed, which is similar to the morphology of previously reported cell-derived vesicles.[23] The protein components of the platelet lysates, VEs, and Act-VEs were determined by Coomassie staining after polyacrylamide gel electrophoresis (Figure 2d). There was no significant difference between VEs and Act-VEs, whereas the protein patterns of the platelets were different from those of VEs. The formulated Act-VEs showed good colloidal stability in PBS solution for at least 4 days (Figure 2e). To optimize the incubation time for aggregate formation, Act-VEs were incubated for different time intervals at 37 °C incubation. As shown in Figure S1c, Supporting Information, Act-VEs formed large sizes within 0.5 h, and the size did not change significantly until 48 h. Considering that VEs formed similar large aggregates after

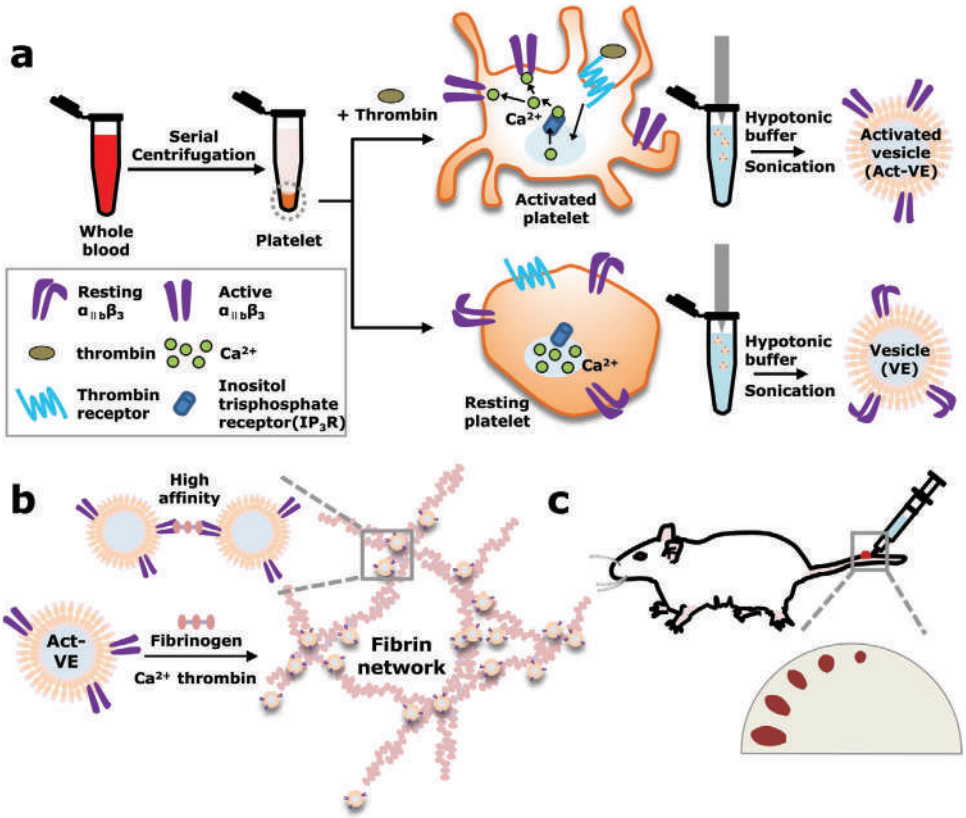

Figure 1. a) Schematic illustration for the preparation of Act-VEs and b) in vivo hemostatic activity after injection of Act-VEs intravenously.

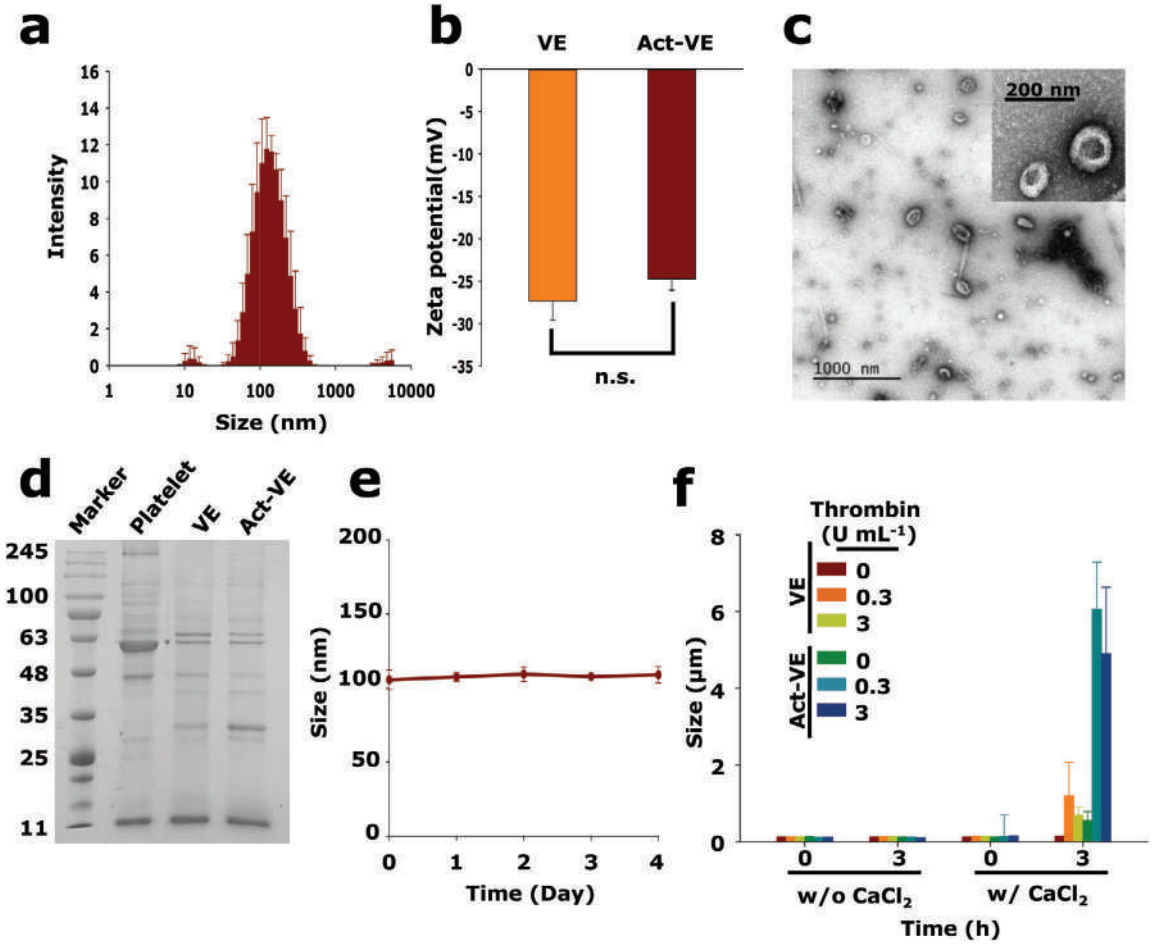

Figure 2. a) Hydrodynamic diameter and b) surface zeta potential of Act-VEs measured by DLS. c) TEM image of Act-VEs. d) Polyacrylamide gel electrophoresis of platelet lysate, VEs, and Act-VEs stained with Coomassie blue. e) Particle size of Act-VEs at 4 °C for 4 days in PBS solution. f) Hydrodynamic size of VE and Act-VE aggregates in the presence of thrombin and $CaCl_2$ after incubation at 37 °C.

24 h under the same conditions in our previous study, aggregation was observed in Act-VEs at a significantly earlier time point compared to VEs.[22] This result might be attributed to the high amount of active αIIbβ3 on Act-VEs for preferred interaction with fibrin and other components.[8] To compare the aggregation behavior of VEs and Act-VEs, each vesicle was incubated for 3 h with thrombin in the absence and in the presence of $CaCl_2$ (Figure 2f). The sizes of aggregates by VEs and Act-VEs at a thrombin concentration of 0.3 U mL^{-1} were 1.20 ± 0.87 and 6.06 ± 1.22 μm, respectively. This result indicates that Act-VEs could form significantly larger aggregates than VEs.

To examine whether Act-VEs could form dense and large fibrin clots or not, fibrinogen was incubated with platelets, VEs, and Act-VEs for 40 min. The resulting fibrin clots were visualized by confocal microscopy and SEM respectively. For fluorescence labeling of fibrin clots, fibrinogen-FITC was incubated with the platelets, VEs, and Act-VEs. As shown in the upper panel in **Figure 3**, fibrinogen showed poor network formation of fibrin after 40 min incubation. However, much stronger fluorescence signals of cross-linked fibrin networks were observed in Act-VEs, compared to platelets and VEs. The lower panel in Figure 3 shows SEM images of fibrin networks by the three samples. Although microsized platelets were observed in the SEM images, nanosized VEs and Act-VEs were not observed. Act-VEs formed much denser fibrin networks than platelets and VEs, which is consistent with the fluorescence images (upper panel in Figure 3).

All animal care and experimental procedures were approved by the Animal Care Committee of Konkuk University. Before in vivo administration, biocompatibility of Act-VE was examined for fibroblast cell line, L929 cells. As shown in Figure S1d, Supporting Information, cell viability was not significantly changed after treatment of Act-VEs up to the concentration of 100 μg mL^{-1}. This result indicates that Act-VE has negligible toxicity to normal murine fibroblasts. After intravenous injection, disruption of the vascular structures resulted in lots of bleeding.[24] To demonstrate the hemostatic activity of Act-VEs in the injured tail vein, platelet, VEs, and Act-VEs were injected intravenously and the amount of bleeding was monitored by soaking up blood with filter papers, as previously reported.[25–27] **Figure 4**a shows images of blood on filter paper at predetermined time intervals. Act-VEs resulted in a low amount of bleeding, whereas the control and platelets showed lots of bleeding. The bleeding time after intravenous injection is shown in Figure 4b. The bleeding time of the control, platelets, VEs, and Act-VEs were 25.90 ± 4.89, 23.00 ± 4.76,

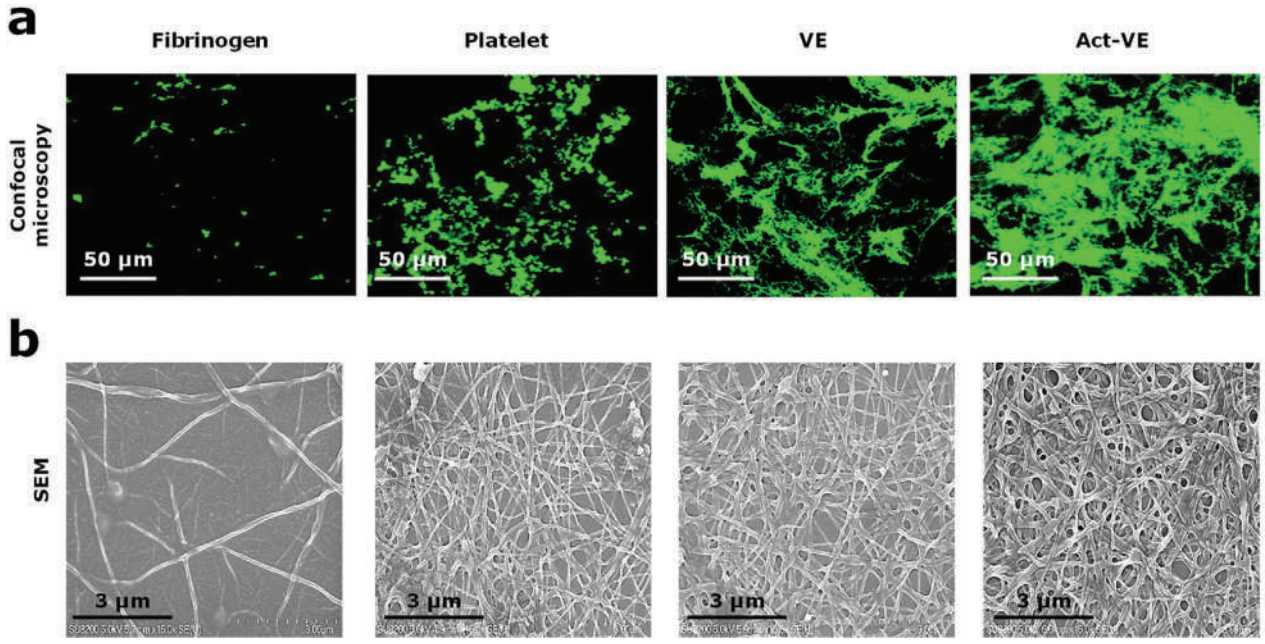

Figure 3. Fibrin networks examined by a) confocal microscopy imaging of fluorescently labeled fibrin network (scale bars = 3 μm) and b) SEM (scale bars = 50 μm).

18.00 ± 2.65, and 14.50 ± 5.80 s, respectively. This result demonstrates that Act-VEs allowed fast hemostasis of the injured vessel compared to the control and platelets. This prompt hemostasis might lessen the amount of bleeding at wound sites via favorable formation of fibrin networks through endogenous platelets and Act-VEs. It should also be noted that previously developed Quik-Clot showed heat-induced tissue damages during absorption of water and positively charged ions for coagulation.[28] However, Act-VEs provide promising hemostatic activity by interaction between Act-VEs and diverse coagulation factors, for example, Ca^{2+} and thrombin.

In previous studies, cytokines from activated platelets elicited strong inflammation, which is closely related to inflammatory diseases, for example, rheumatoid arthritis, osteoarthritis, and atherosclerosis.[29–31] Accordingly, efficient hemostatic biomaterials that do not release pro-inflammatory cytokines are needed. IL-1β is a representative pro-inflammatory cytokine that could trigger endothelial inflammation. Activated platelets induce a nuclear factor kappa B (NF-*K*B) signaling pathway and permeability of endothelial cells, resulting in the recruitment of immune cells.[31,32] Figure 4c shows IL-1β released from platelets and Act-VEs after incubation in culture media for 4 days. The level of released IL-1β from platelets and Act-VEs were 35.14 ± 4.69 and 4.84 ± 4.86 pg mL^{-1}, respectively. This result clearly demonstrates that Act-VEs exhibited, reduced the release of IL-1β compared to platelets. In our previous study, VEs also showed significantly reduced IL-1β release around 4.4 pg mL^{-1}, which is similar to Act-VEs.[22] Considering that platelet-derived vesicles showed rapid clearance within 30 min for mouse and 10 min for rabbit after injection in previous study, it is conceivable that Act-VEs could also be cleared safely out from body after injection.[20,33] However, several additional

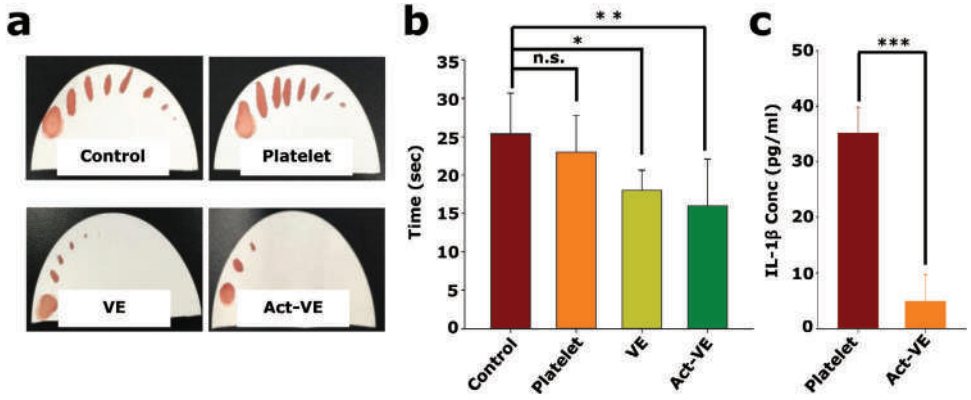

Figure 4. a) Blood-dotted filter papers of injured vein and b) tail-bleeding time after injection of PBS solution (control), platelets, VEs, and Act-VEs; *$p < 0.05$, **$p < 0.01$; n.s, not significant. Error bars mean standard deviations (n = 3–10). c) Amount of IL-1β released from activated platelets and Act-VEs in the presence of thrombin; ***$p < 0.001$.

animal studies are still needed including examination of potential thrombin throughout the body and time course safety study for the practical application of these Act-VEs.

In this study, we developed Act-VEs from thrombin-activated platelets as an efficient hemostatic biomaterial. The Act-VEs exhibited improved clot formation and contributed to faster aggregation, which might be due to active $\alpha\|b\beta3$ on the membrane of Act-VEs. In addition, the hemostatic ability of Act-VEs was superior to that of platelets in the mouse tail-bleeding assay in vivo. Act-VEs released lower levels of pro-inflammatory cytokine (IL-1β) compared to activated platelets. Because Act-VEs could provide promising hemostatic ability and release low levels of IL-1β, they could serve as an alternative to hemostatic materials. In addition, this result indicates that activation of platelets is a crucial step for the design of platelet-derived biomaterials for efficient hemostatic activity.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

activated platelet-derived vesicles, fibrin clot, hemostasis, inflammatory cytokine, platelet activation, platelet-derived vesicles

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