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# Procoagulant platelets: Generation, characteristics, and therapeutic target

Yaxin Chu[1] | Han Guo[1] | Yuncong Zhang[2] | Rui Qiao[1]

[1]The Department of Laboratory Medicine, Peking University Third Hospital, Beijing, China

[2]The Department of Laboratory Medicine, Peking University International Hospital, Beijing, China

**Correspondence**
Rui Qiao, Peking University Third Hospital, 49 North Garden Road, Haidian District, Beijing 100191, China.
Email: qiaorui@bjmu.edu.cn

## Abstract

Platelets play a pivotal role in hemostasis. Activated platelets are classified into two groups, according to their agonist response: aggregating and procoagulant platelets. Aggregating platelets consist of activated integrin $\alpha IIb\beta3$ and stretch out pseudopods to further attract platelets to the site of injury by connecting with fibrinogen. They mainly gather in the core of the thrombus and perform a secretory function, such as releasing adenosine diphosphate (ADP). Procoagulant platelets promote the formation of thrombin and fibrin by interacting with coagulation factors and can thus be considered as the connector between primary and secondary hemostasis. In addition to their functions in blood coagulation, procoagulant platelets play a proinflammatory role by releasing platelet microparticles and inorganic polyphosphate. Considering these important functions of procoagulant platelets, this subpopulation warrants detailed study to analyze their potential in preventing human diseases. This review summarizes the generation and important characteristics of procoagulant platelets, as well as their potential for preventing the adverse effects associated with current antiplatelet therapies.

**KEYWORDS**
aggregating platelets, coagulation, hemostasis, procoagulant platelets, proinflammatory

## 1 | INTRODUCTION

Hemostasis is a critical physiological process that stops bleeding at the site of a vascular injury and maintains the integrity of vessels. Cooperation between platelets, coagulation factors, and endothelial cells is critical to this complex process. Hemostasis is initiated by wounded vessels, which have exposed extracellular matrix, and further activates platelet adhesion, aggregation, secretion, and a cascade of coagulation factors. Platelet activation and the coagulation system function in concert to seal the damaged site; thus, their imbalance results in bleeding or thrombosis.

The way in which platelets interact with coagulation factors and accurately localize to the damaged site is of considerable interest.

Activated platelets can be classified into at least two forms, procoagulant and aggregating platelets.[1–5] These two subpopulations have different manifestations. For example, the most abundant integrin, $\alpha IIb\beta3$, exhibits an "open" form on aggregating platelets and a "closed" form on procoagulant platelets, which display exposed phosphatidylserine (PS).[6] Aggregating platelets contain numerous pseudopods, whereas procoagulant platelets are balloon-shaped without pseudopods.[3,6] In addition, aggregating platelets strongly secrete bioactive molecules such as ADP, adenosine triphosphate (ATP), and serotonin, whereas the secretory function of procoagulant platelets is weak.[6] While the exact role of aggregating platelets in inflammation is not yet completely understood, procoagulant platelets are known to exert proinflammatory effects and can

release inflammation mediators such as inorganic polyphosphate and microparticles.[7-10] We show the morphological differences between resting platelet, aggregating platelet and procoagulant platelet in Figure 1.

In this review, we focus on the function of procoagulant platelets in recruiting coagulation factors to the damaged site and serving as a bridge between primary and secondary hemostasis.[7,11] First, we summarize the mechanisms of procoagulant platelet formation. Second, we discuss the attributes pivotal to procoagulant platelets, and finally, we highlight recent studies on their therapeutic use as clinical biomarkers.
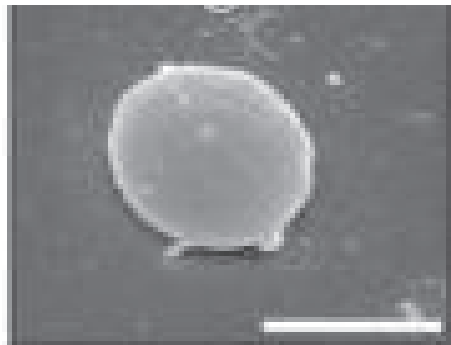


**(A)**
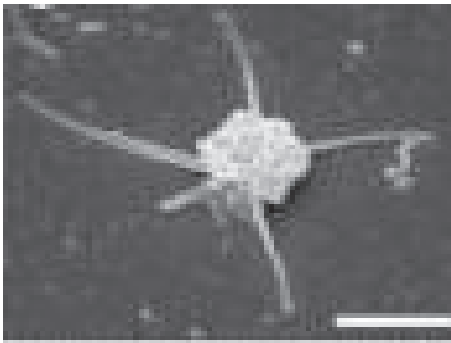


**(B)**



**(C)**

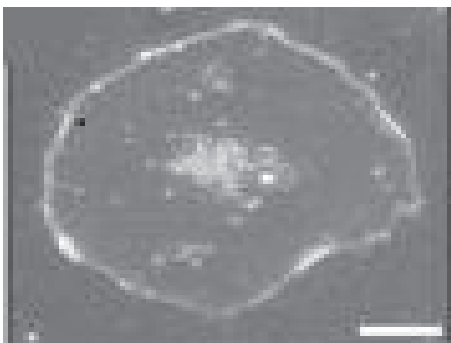**FIGURE 1** Different states of platelets viewed using scanning electron microscopy. A, Resting platelets are smooth and flat. B, Aggregating platelets contain many pseudopods. C, Procoagulant platelets are characterized by balloon-shaped morphology and do not contain pseudopods. Bar = 2 μm. Images were taken from Agbani et al[11]

## 2 | MECHANISMS OF PROCOAGULANT PLATELET FORMATION

Procoagulant platelets are generally considered to be apoptotic.[12-14] However, recent studies have revealed that procoagulant platelets undergo necrosis. The suppression of caspase, a modulator of apoptosis, does not affect the procoagulant function of platelets,[15,16] whereas the inhibition of cyclophilin D (CypD), which modulates necrosis by inducing the opening of mitochondrial permeability transition pores (mPTP), greatly reduces their procoagulant function.[17] Jackson et al[18] compared the characteristics of apoptosis, necrosis, and procoagulant platelets (summarized in Table 1). However, upon inhibiting CypD, procoagulant platelets are still formed. In a study in CypD knockout mice, platelets externalized PS, and there were no major differences in the tail transection bleeding times between $CypD^{+/+}$ and $CypD^{-/-}$ mice.[17] These findings suggest that other pathways may be involved in the generation of procoagulant platelets.

Platelets vary in their responses to agonist stimulation, and various multifactorial processes determine the fate of platelets. Rheological and topographical factors within the thrombi in vivo have been proposed to affect the heterogeneity in platelet activation states.[19] Fluid dynamic studies have shown that platelets within the thrombus core undergo longer exposure to solute agonists than those on the outer thrombus shell. The thrombus core is less porous and has stronger platelet–cell adhesion than the outer thrombus shell, where convective flow currents and the relative increase in permeability result in decreased exposure to agonists.[20] Environmental factors such as different adhesive surfaces, and intrinsic platelet characteristics, such as age, surface protein expression, and subcellular components, may play a role in determining the heterogeneity in platelet response after activation.[20,21]

In the context of those undergoing necrosis, we describe the classical mechanisms underlying the formation of procoagulant platelets. Detailed mechanisms can be found in Figure 2.

### 2.1 | Agonist demand

Although many agonists can activate platelets, only strong agonists can induce the formation of procoagulant platelets. Agonists include physiological and non-physiological agonists. Among the physiological agonists, the co-stimulation of thrombin or thrombin receptor-activating peptide and glycoprotein VI agonists such as collagen, convulxin, and collagen-related peptides, along with calcium ($Ca^{2+}$) in the solution, produces the maximum activation effect.[11,22–24] ADP had little effect on the generation of procoagulant platelets according to Pasalic et al[23] Hua et al[11] evaluated the proportion of procoagulant platelets upon treatment with various agonists, including thrombin, collagen, or thrombin plus collagen, and showed that co-stimulation with collagen and thrombin helped achieve the highest procoagulant platelet ratio (59.21%), followed by collagen activation alone (45.76%), with thrombin activation the least effective (20.78%). However, Pasalic et al[23] found that co-stimulation with collagen

**TABLE 1** Characteristics of procoagulant platelets during apoptosis and necrosis (summarized from Jackson et al[18])

| Characteristics | Apoptosis | Necrosis | Procoagulant platelets |
|---|---|---|---|
| Stimulus | Ratio of $Bcl_{xl}$ to Bax/Bak | Cyclophilin D | Activation of GP VI receptor and/or protease-activated receptors (e.g., thrombin, collagen, collagen-related peptide) |
| $Ca^+$ Dependence | Independent of $Ca^+$ | Dependent on $Ca^+$ | Dependent on $Ca^+$ |
| Morphology | Cell shrinkage and apoptotic body formation | Cell swelling | Cell swelling and ballooning |
| Membrane integrity | Loss in the late stage | Loss in the early stage | Loss of membrane integrity |
| Mitochondrial transmembrane potential ($\Delta\Psi m$) | Loss in the late stage | Loss in the early stage | Loss of $\Delta\Psi m$ |
| PS exposure | Exposed in the late stage | Exposed in the early stage | Exposed |
| Formation of MPTP | No | Yes | Yes |
| Method of inhibition | Bax/Bak knockout | Cyclophilin D knockout | Cyclophilin D knockout |

Abbreviations: GP VI, glycoprotein VI; mPTP, mitochondrial permeability transition pore; PS, phosphatidylserine.

and thrombin had the highest efficiency in producing procoagulant platelets, regardless of the sample type. Compared with the results of Hua et al[11], those of Pasalic et al[23] indicated that thrombin was more efficient than collagen in generating procoagulant platelets. Pasalic et al[23] also compared washed platelets, platelet-rich plasma, and whole blood, in terms of procoagulant platelet generation, and found that whole blood is the preferred sample type, whereas washed platelets are not ideal, as there are fewer steps, such as washing and centrifugation, required when whole blood samples are used compared to those when washed platelets are used.

In our laboratory, we have attempted to detect pro-platelets using whole blood samples and observed that a high number of pre-treatment steps can lead to excessive activation and loss of platelets. We detected the platelets using forward scattering light, side scattering light (SSC), and CD41a, a specific marker for platelets, and defined procoagulant platelets as those that were positive for the CD62P and GSAO [4-(N-(S-glutathionylacetyl)amino)phenylarsonous acid] markers for necrotic platelets (R. Qiao, 2021, unpublished data).

Among non-physiological agonists, $Ca^{2+}$ ionophores, such as A23187 or ionomycin, have been used to induce a high and sustained rise in cytosolic $Ca^{2+}$.[22] A previous study showed that almost all platelets can become procoagulant upon $Ca^{2+}$ ionophore treatment. The methods of detecting procoagulant platelets in vitro employed in several studies, including the types of samples, agonist types and concentration, activation time, and the ratio of procoagulant platelets, are summarized in Table 2.

Tan et al[25] measured the percentage of procoagulant platelets in whole blood using CD45, a specific marker of leukocytes, CD41a, CD62P, and GSAO. Platelets that take up GSAO and express surface P-selectin represent the procoagulant subpopulation (Figure 3).

## 2.2 | $Ca^{2+}$ dependency

Cytosolic $Ca^{2+}$ concentration is increased in "procoagulant platelets" and "non-procoagulant platelets," which are both activated platelets. The key determinant of procoagulant platelets is maintained, extremely high cytosolic $Ca^{2+}$ levels. The process by which this can be attained can be divided into three stages.

First, through intracellular release and extracellular entry, cytosolic $Ca^{2+}$ concentration is increased, with the endoplasmic reticulum (ER) having the highest $Ca^{2+}$ concentration among the cellular organelles. Three important molecules, stromal interaction molecule 1 (STIM1), Orai1, and TRPC6, are involved in extracellular $Ca^{2+}$ entry.

### 2.2.1 | STIM1

STIM1, a type 1A transmembrane $Ca^{2+}$ sensor in the ER of platelets, can detect the release of $Ca^{2+}$ from intracellular stores and regulate Orai1.[4,26] STIM1 senses the $Ca^{2+}$ content of intracellular stores using a $Ca^{2+}$-binding EF-hand domain that protrudes into the lumen of the store. Upon store depletion, $Ca^{2+}$ is released from the EF-hand domain, and the plasma membrane store-operated calcium entry (SOCE) channel is activated. Varga-Szabo et al[27] reported that STIM1 deficiency markedly reduced $Ca^{2+}$ entry, and mice deficient in STIM1 showed severe defects in thrombus formation under flow, but only had a limited risk of bleeding complications. In addition, SOCE channels in mice carrying the STIM1 mutant were permanently opened in vivo, which led to sustained $Ca^{2+}$ influx from the extracellular space and a bleeding phenotype in mice in vitro.[28] These findings indicate the key regulatory role of STIM1 in maintaining $Ca^{2+}$ levels via SOCE channels in platelets.
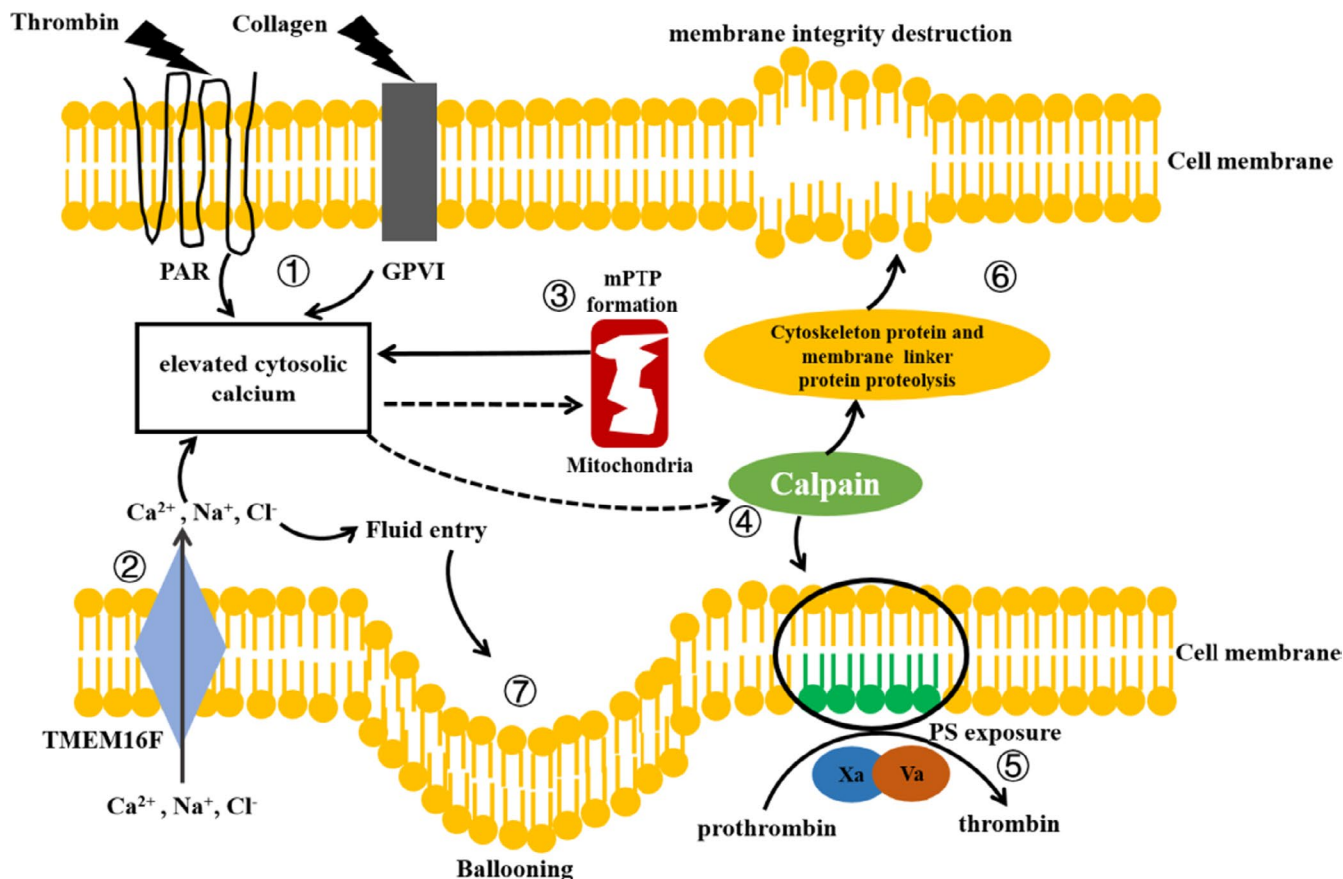
**FIGURE 2** Mechanisms of procoagulant platelet formation. (1) Protease-activated receptor and glycoprotein VI are located on the surface of platelets. After stimulating the platelets with agonists, such as thrombin and collagen, cytosolic $Ca^{2+}$ concentration increases. (2) TMEM16F located on the platelet's membrane allows the entry of $Na^{+}$, and $Cl^{-}$, and especially $Ca^{2+}$. This mechanism, to some extent, promotes higher $Ca^{2+}$ concentration. (3) Elevated cytosolic $Ca^{2+}$ concentration triggers the formation of mPTPs, which in turn leads to the release of $Ca^{2+}$ stored in the mitochondria. (4) Elevated cytosolic $Ca^{2+}$ activates calpain, which causes the redistribution of phospholipids and PS exposure. (5) PS exposure is a prerequisite for procoagulant activity. Coagulation factors Xa and Va aggregate on the PS surface and convert prothrombin into thrombin. (6) Activated calpain also hydrolyzes cytoskeleton proteins and membrane linker proteins, which damages the membrane integrity. (7) The entry of salt ions into platelets leads to high osmotic pressure and further fluid entry, thereby resulting in ballooning

## 2.2.2 | Orai1 and TRPC6

Extracellular $Ca^{2+}$ ions mainly enter through the plasma membrane channels Orai1 and TRPC6, which are the most abundant channels on the platelet surface.[26,29] Orai1 is a type of SOCE channel.[26] Using Synta-66, an Orai1 blocker, Abbasian et al[29] found that the percentage of procoagulant platelets and the median fluorescence of Fluo-4, which has high affinity for $Ca^{2+}$, was reduced in whole platelets. The median fluorescence of Fluo-5 N, which has a low affinity for $Ca^{2+}$, in procoagulant platelets was unaffected. The authors speculated that Orai1 contributes to the elevation of cytosolic $Ca^{2+}$ but is not directly involved in the generation of supramaximal $Ca^{2+}$.[24]

TRPC6 is an unselective cation channel. Upon platelet activation, $Na^{+}$ enters the platelets via TRPC6, and $Ca^{2+}$ entry occurs subsequently via $Na^{+}/Ca^{2+}$ exchange. SOCE channels, especially the major SOCE channel Orai1, have been proposed to be potential antithrombotic targets.[28,30] However, such targeting has been associated with

serious adverse effects, such as severe combined immune deficiency in patients with a missense mutation in Orai1.[31] To address this problem, individualized genetic testing should be performed in patients carrying a missense mutation in Orai1.

The second stage is the entry of cytosolic $Ca^{2+}$ into the mitochondria, and the opening of mitochondrial permeability transition pores (mPTPs). Because the cytosolic $Ca^{2+}$ concentration in the first stage is different in procoagulant and non-procoagulant platelets, their mitochondrial $Ca^{2+}$ concentration also varies. $Ca^{2+}$ enters the mitochondria through the mitochondrial calcium uniporter (MCU).[32] The pharmacologic inhibition of MCU might effectively abrogate the formation of procoagulant platelets without affecting other aspects of platelet activation, thus representing a novel pharmacologic target specific to the prevention of procoagulant platelets without affecting normal hemostasis.[33] Similar to the formation of action potentials, which are activated only when stimuli reach or exceed the threshold, mPTP opening also has a threshold. mPTPs open only when $Ca^{2+}$ uptake into mitochondria reaches this threshold; thus,

TABLE 2 Methods used to generate procoagulant platelets described in the literature

| Sample types | Agonists | Conditions | Procoagulant platelet ratio |
|---|---|---|---|
| Washed platelets (5 × 10$^4$/μL)[7] | Thrombin (0.1 U/ml) | 37°C, 10 min | 59.2% |
| | Collagen (5 μg/ml) | 37°C, 10 min | 45.76% |
| | Thrombin (0.1 U/ml) + collagen (5 μg/ml) | 37°C, 10 min | 20.78% |
| Whole blood[25] | Thrombin + collagen (final concentrations are variable) | Room temperature [25–27°C], 10 min | 29.7% |
| Whole blood[23] | Thrombin (2 U/ml) | Room temperature [25–27°C], 10 min | 13.15% |
| | Collagen (10 μg/ml) | Room temperature [25–27°C], 10 min | 1.2% |
| | Thrombin (2 U/ml) + collagen (10 μg/ml) | Room temperature [25–27°C], 10 min | 17.3% |
| Whole blood (5 × 10$^4$/μl)[29] | Thrombin (2 U/ml) + collagen (10 μg/ml) | 37°C, 10 min | 60% |
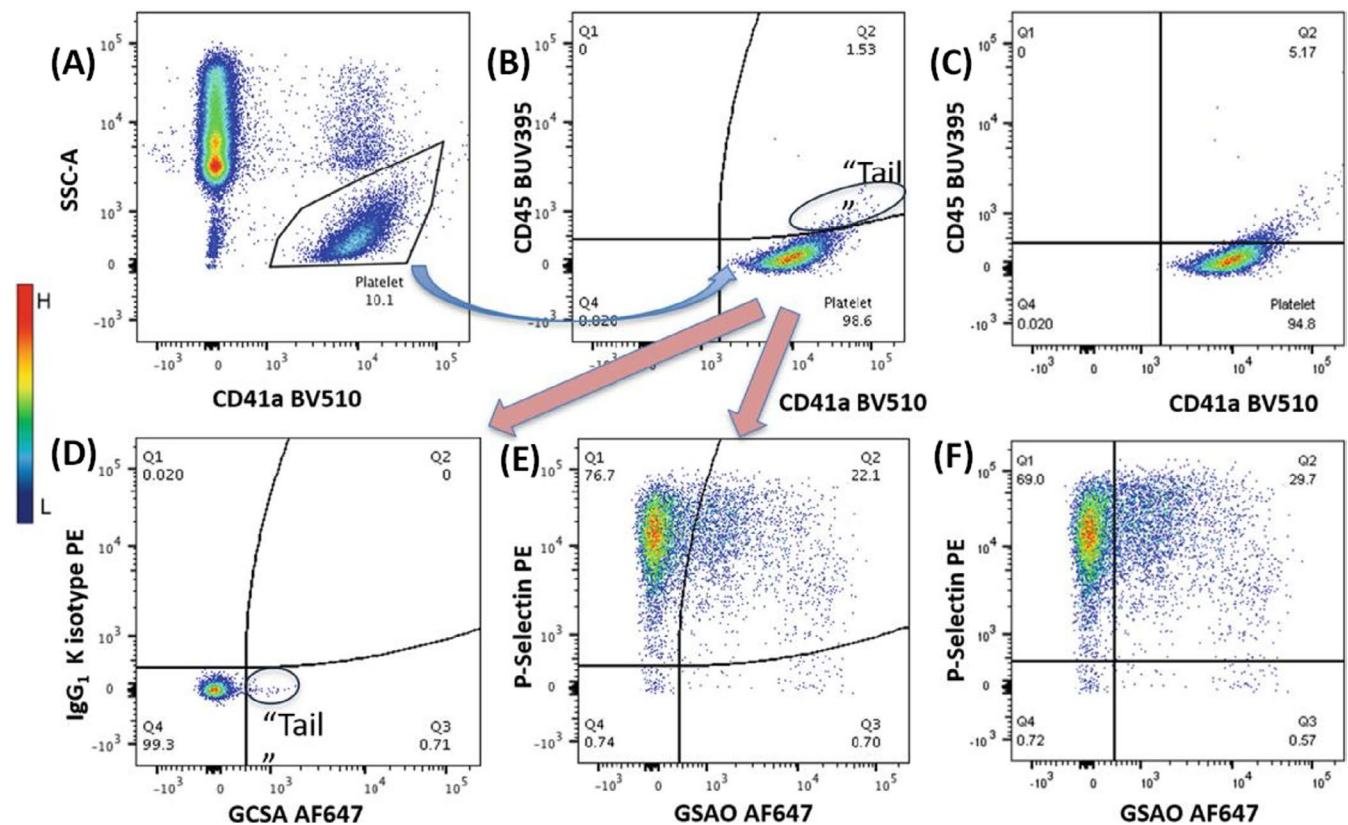


FIGURE 3 Detection of procoagulant platelets using flow cytometry. A, Platelets are first identified using polygonal gating on the SSC-A versus CD41a plot and as presenting low SSC and CD41a positivity, and subsequent analyses are based on this gate. B, Gating is further refined in the lower right quadrant by selecting CD41a$^+$/CD45$^-$ cells using curved quadrants, and subsequent analyses are based on this gate. The "tail" population (CD41a$^+$/CD45$^+$) is excluded, as it represents aggregates of platelets and leukocytes. C, Applying straight quadrants to the same platelet population and thresholds for both axes shown in (B) yields slightly different results. D, Thresholds for CD62P and GSAO are set by PE and GSCA isotype controls. E, Procoagulant platelets are identified in the upper-right quadrants as CD62P$^+$/GSAO$^+$ cells in a stimulated sample. F, Applying straight quadrants to the same platelet population and thresholds for both axes shown in (E) yields slightly different procoagulant platelet percentages. Images were taken from Tan et al[25]

below the threshold of mPTP opening, platelets do not possess procoagulant properties.

In the third stage, mPTP opening leads to the cytoplasmic transport of mitochondrial $Ca^{2+}$, generating extremely high cytosolic $Ca^{2+}$ concentrations and subsequently, the formation of procoagulant platelets.[12,29,33]

In summary, we speculate that mPTP opening, which leads to a high cytosolic $Ca^{2+}$ concentration, is crucial for procoagulant platelet formation. Therefore, we can describe procoagulant platelet formation as an "all-or-nothing" process. Increased cytosolic $Ca^{2+}$ concentration is required for the formation of both procoagulant and non-procoagulant platelets. In particular, higher and more sustained cytosolic $Ca^{2+}$ concentration generates procoagulant platelets, and lower and less sustained cytosolic $Ca^{2+}$ concentration generates non-procoagulant platelets.

## 2.3 | TMEM16 and chloride ions ($Cl^-$) in water entry

The transmembrane protein TMEM16 is also called anoctamin (ANO). TMEM16A (ANO1), TMEM16B (ANO2), and TMEM16F (ANO6) are the most studied members of the TMEM16 family.

### 2.3.1 | TMEM16A and TMEM16B

$Cl^-$ blockers are used to inhibit $Cl^-$ entry in physiological agonist-activated platelets. $Cl^-$ entry via $Cl^-$ channels is speculated to be necessary for membrane hyperpolarization by providing the driving force for $Ca^{2+}$ entry and triggering full PS exposure.[34] TMEM16A and TMEM16B are $Ca^{2+}$-activated $Cl^-$ channels (CaCCs).[35] Increased cytosolic free $Ca^{2+}$ triggers the opening of CaCCs and allows the entry of $Cl^-$ through these channels. In turn, increased anion concentration inside cells promotes $Ca^{2+}$ entry into cells.

### 2.3.2 | TMEM16F

To date, there are three known functions of TMEM16F. In 2012, it was described as a $Ca^{2+}$-activated channel for the entry of cations such as $Ca^{2+}$, $Na^+$, $Li^+$, $K^+$, $Rb^+$, $Ba^{2+}$, and $Cs^+$, with increased permeability to $Ca^{2+}$ and $Ba^{2+}$.[36] Second, TMEM16F was described as a $Ca^{2+}$-activated channel for the entry of anions, such as $Cl^-$. Lastly, it was also denoted as a $Ca^{2+}$-activated phospholipid scramblase that translocates PS to the platelet surface, thus facilitating blood coagulation.[35–38]

There are two major types of phospholipid scramblases: caspase-dependent lipid scramblase, such as Xkr8, that induce PS externalization, and $Ca^{2+}$-activated phospholipid scramblase, that mediates PS externalization in response to an intracellular increase in $Ca^{2+}$.[39] Yang et al[35] generated *TMEM16F* knockout mice and reported that TMEM16F is important for thrombin generation and

the activity of $Ca^{2+}$-activated scramblase in blood cells. *TMEM16F* knockout mice not only had impaired hemostasis as evidenced by a prolonged bleeding time, but also had impaired carotid artery thrombus formation, revealing the crucial role of TMEM16F in thrombosis.[4,35]

In 1979, the first patient with Scott syndrome, a rare bleeding disorder, was reported. The patient had a normal platelet level, structure, adhesion, secretion, and aggregation, while a notable defect in procoagulant activity was observed. This phenomenon was due to decreased $Ca^{2+}$-dependent PS exposure, which is regulated by TMEM16F.[38] To date, only six cases of Scott syndrome have been reported.[40] TMEM16F may serve as a new pharmaceutical target to treat human hemostatic and thrombotic disorders, such as stroke and heart attack.[37]

### 2.3.3 | Water entry

The entry of salts, such as $Ca^{2+}$ and $Cl^-$, provides an osmotic drive for water entry and subsequently causes ballooning, a characteristic of procoagulant platelets. Agbani et al[7] investigated whether water entry was required for ballooning. Using sucrose to increase extracellular osmolality, they reported the blocking of water entry and ballooning along with significant inhibition of thrombus formation.

## 2.4 | Role of calpain

Sustained elevation of cytosolic $Ca^{2+}$ concentration can activate the thiol protease calpain. Calpain hydrolyzes membrane linker proteins, such as actin, vinculin, and myosin, which cause cytoskeleton remodeling and disruption and PS exposure.[41,42] Calpain also participates in the release of microparticles.[1,42] Microparticles, the "dust" of platelets, possess higher procoagulant activity than platelets, carry inflammatory substances, and can be delivered remotely.

## 3 | FEATURES OF PROCOAGULANT PLATELETS

### 3.1 | PS exposure

A key characteristic of procoagulant platelets is PS exposure, which is necessary for coagulation assembly and thrombin generation. In normal resting platelets, the phospholipid bilayer of the cell membrane is asymmetric, comprising phosphatidylcholine and sphingomyelin mainly on the outer surface, whereas PS and phosphoethanolamine are located on the inner part. Two enzymes maintain this asymmetry: flippase transports lipids from the outer surface to the inner surface, whereas floppase has the opposite activity.[43] PS exposure is dependent on the $Ca^{2+}$-dependent scramblase and calpain.[1,37,39,41]

## 3.2 | Balloon-like membrane morphology

Ballooning is one of the properties of procoagulant platelets. Ballooning is an irreversible process that increases the area for coagulation assembly [for example, prothrombinase complex (FXa-FVa) and tenase complex (FIXa-FVIIIa)]. Ballooning also affects PS exposure, and the release of microvesicles decreases when ballooning is inhibited.

## 3.3 | Glycoprotein IIb/IIIa (GPIIb/IIIa) inactivation

Platelets in the thrombus can be aggregating or procoagulant platelets. The former subpopulation participates in the retraction of fibrin clots, which require fibrin formation and integrin "outside-in" signaling. During signaling, aggregating platelets possess an activated GPIIb/IIIa. Owing to ballooning and cytoskeleton disruption, procoagulant platelets lose their adhesive function, showing "closed" GPIIb/IIIa.[4]

The presence of an activated GPIIb/IIIa conformation is confirmed by PAC-1 antibodies for human platelets.[4] Anna et al utilized PAC-1, which can bind the active conformation of GPIIb/IIIa, to detect aggregatory platelets. PAC-1 binds to or near to the fibrinogen site, and its affinity to the active GPIIb/IIIa conformation is higher than that to fibrinogen. Munnix et al[6] found that platelets that bind annexin A5, a marker for PS detection, failed to bind PAC-1. They concluded that the manifestation is reasonable because the round morphology and lack of pseudopods is compatible with a low integrin activation state, and thus diminished adhesion. This generates a balance, in which procoagulant activity suppressed integrin activation, whereas aggregate formation reduced the procoagulant activity of platelets. After activation, normal-sized platelets bound PAC-1 with a negative combination of annexin V, a marker for procoagulant platelets that binds to PS, and were defined as "aggregatory." The authors speculated that the reduction in PAC-1 binding in PS-positive platelets is due to the down-regulation of fibrinogen receptors via the reduction in aggregatory response.[7,11]

## 3.4 | Procoagulant spreading

The link between ballooning and procoagulant spreading was first identified in 2015.[7] In this study, most ballooning platelets had spread membrane structures, and all procoagulant spreading platelets had ballooned. This observation suggests that ballooning is required for procoagulant spreading but not vice versa.[7] However, procoagulant spreading only occurs when platelets adhere to agonist-coated surfaces, and those in suspension did not exhibit procoagulant spreading.[7,15]

## 3.5 | Platelet-derived microparticles

Microparticles ranging between 0.1 and 1 μm are derived from various cell types in the plasma. Platelets are the major source of microparticles in circulation.[44] The formation of microparticles is a consequence of membrane remodeling.[2] Through phospholipid scramblases and calpain, procoagulant platelets release platelet-derived microparticles (PMPs).[7] Proteins that mediate platelet function related to hemostasis, inflammation, and immunity are also found in platelet PMPs. PMPs serve as a surface for the assembly of tenase and prothrombinase complexes to boost thrombin formation and coagulation.[45] The coagulation capacity of PMPs is suggested to be 50- to 100-fold higher than that of activated platelets.[46] Platelets also express inflammasomes, that can hydrolyze and release inflammatory mediators, such as IL-1α and IL-1β, which are mostly wrapped in PMPs.[47] PMPs can then interact with inflammatory leukocytes, such as monocytes and neutrophils, and induce endothelial cells to express adhesion molecules, such as intercellular cell adhesion molecule-1 (ICAM-1). All these effects trigger an inflammatory response.[48,49]

## 3.6 | Release of polyP

Platelet dense granules contain polyP, which has a mean chain length of 80 units. Verhoef et al[50] proposed that polyP promotes contact system activation and coagulation. They showed that polyP released from dense granules aggregates into nanoparticles that accumulate on the platelet surface and are of sufficient size to promote FXII activation.[50] Meanwhile, Fredenburgh et al[51] speculated that in addition to FXI and FXII inhibitors, neutralizing agents of polyP attenuate thrombosis without disrupting hemostasis. Long-chain polyP located on the platelet surface initiates FXII contact activation and leads to coagulation, whereas short-chain polyP, which is soluble and does not bind to platelets, has minor effects on FXII activation and may serve other functions, such as interaction with tissue factor pathway inhibitor (TFPI).[52] PolyP has been recognized as a procoagulant platelet-derived inflammatory mediator. PolyP-driven FXII activation causes the promotion of the kallikrein-bradykinin pathway. Following its activation, kallikrein hydrolyzes kininogen to induce bradykinin formation. The binding of bradykinin to its receptor then results in vessel dilation, neutrophil chemotaxis, and increased vascular permeability.[9]

# 4 | THERAPEUTIC APPLICATIONS OF PROCOAGULANT PLATELETS

## 4.1 | Clinical biomarkers

There is always a need for suitable markers and/or identity criteria for the clinical assessment of thrombotic or bleeding events. As procoagulant platelets participate in thrombin generation and hemostasis promotion, altering their proportions may result in varying levels of risk of thrombosis and bleeding. Pasalic et al[23] found that patients with coronary heart disease had a higher platelet procoagulation potential than healthy controls in response to thrombin

plus collagen (18.2 ± 2.2% vs. 25.2 ± 3.9%, $p < 0.05$). Lacunar stroke represents approximately 25% of all ischemic strokes and is associated with smaller infarct size and lower recurrence risk than those of non-lacunar stroke. A previous study showed that patients with lacunar stroke (21.8 ± 11.4%) had the lowest levels of coated platelets (a population of procoagulant platelets) among those with cortical stroke (39.4 ± 12.7%, $p < 0.001$) or controls (31.6 ± 13.2%, $p = 0.008$).[53] In another study, the level of procoagulant platelets (>40%) was associated with recurrent cerebral infarction.[54] A recent study demonstrated that the ability of coated platelets to predict recurrent ischemic events following lacunar stroke (AUC: 0.835 ± 0.08, $p < 0.001$) had a sensitivity of 0.75 (0.35–0.97; 95% CI), specificity of 0.92 (0.85–0.97), positive predictive value of 0.43 (0.26–0.62), and negative-predictive value of 0.98 (0.93–0.99).[55]

Apart from their hemostatic function, procoagulant platelets also exhibit a proinflammatory function. PMPs released primarily by procoagulant platelets are proinflammatory mediators associated with various clinical manifestations. For example, PMPs are elevated in patients with rheumatoid arthritis and infectious diseases, such as HIV, dengue, and malaria. PMPs are also related to disease propagation.[56] Once released, PMPs are widely distributed. Thus, we speculate that procoagulant platelets can be used as biomarkers for inflammatory diseases. Although there are only a few studies on this topic, this is a promising research direction to explore. We can use experimental approaches based on the features introduced above to detect procoagulant platelets in vitro.

## 4.2 | PS exposure is an assistive measure, but not sufficient, for procoagulant platelet detection

Although PS exposure is a prerequisite for the formation of a procoagulant platelet subpopulation, it is not entirely correlated with procoagulant platelets. Along with the imperfect correlation between PS exposure and the presence of coagulation factors on the platelet surface, even after strong activation, only some platelets expose PS, and a small subset of them is procoagulant.[11] Thus, PS exposure alone is not sufficient to conclude that procoagulant platelets will be formed.[25]

## 4.3 | Detecting procoagulant platelets using a combination of GSAO and P-selectin

The combination of GSAO and P-selectin can detect procoagulant platelets.[23,25] GSAO is a cell death marker that can pass through the procoagulant platelet membrane with elevated permeability, covalently bind to proteins with closely spaced dithiols, and resist washout. When tagged with a reporter compound, flow cytometry can analyze procoagulant platelets using a GSAO-fluorescein marker. P-selectin is normally stored in the α-granule in resting platelets, and is a marker of activated platelets, which express surface P-selectin upon activation.

GSAO in combination with P-selectin can discriminate a population of platelets that present necrotic features and, functionally, a procoagulant phenotype. Positive test results for both GSAO and P-selectin define procoagulant platelets.[25] Patients without coronary artery disease (CAD) had a synergistic increase in the ratio of procoagulant platelets when collagen was added to thrombin (thrombin vs. thrombin plus collagen: 12.2 ± 3.4% vs. 18.2 ± 2.2%, $p < 0.05$); however, platelets from patients with CAD reached their maximal procoagulant potential with thrombin stimulation alone, and no further increase was observed upon the addition of collagen, suggesting that patients with CAD have hypersensitivity to thrombin.[23]

## 4.4 | A novel target for antithrombosis

Antiplatelet drugs are used to treat thrombotic diseases. For example, aspirin, which blocks thromboxane A2 (TXA2) formation, is usually prescribed to prevent cardiovascular diseases, and $P2Y_{12}$ inhibitors such clopidogrel, prasugrel, and ticagrelor, are used to block the secretion of ADP and ATP from platelets. Dual antiplatelet therapy, which refers to the combination of aspirin with a $P2Y_{12}$ inhibitor, is the current treatment for the secondary prevention of atherothrombotic events in patients with acute coronary syndrome or those undergoing elective percutaneous coronary intervention. Aspirin and $P2Y_{12}$ inhibitors resemble GPIIb/IIIa inhibitors such as abciximab and eptifibatide, and target aggregating platelets.[3] However, their clinical applications remain limited, because the inhibition of aggregating platelets is usually accompanied by bleeding, which can range from serious events, such as intracranial hemorrhage, to minor skin bruising.[1] More than 25% of patients using antiplatelet drugs experience ischemic events.[3]

Targeting aggregating platelets corresponds to blocking platelet thrombus formation; thus, the bleeding site cannot be effectively blocked. When procoagulant platelets are targeted, thrombin formation can be inhibited without affecting the function of aggregating platelets, indicating its potential use as an antithrombotic method without bleeding risk. However, there is a lack of drugs that specifically target procoagulant platelets. A study on aquaporin-1 (*AQP1*) knockout mice found that: (1) AQP1 is a critical mediator that regulates procoagulant platelet formation; (2) AQP1 inhibition decreases procoagulant spreading, microvesiculation, PS exposure, and thrombus formation time; and (3) AQP1 inhibition has minimal effects on the secretion and aggregation of platelet granules. These results suggest that AQP1 antagonists may represent novel antithrombotics targeting procoagulant platelets.[57] Unfortunately, there are still no AQP1-specific inhibitors. Carbonic anhydrase (CA) inhibitors, such as acetazolamide and methazolamide, are clinically used as mild diuretics, and may be potent anti-procoagulant agents.[58] Acetazolamide and methazolamide attenuate intracellular $Cl^-$ entry and suppress the procoagulant response of activated platelets *in vitro* and thrombosis *in vivo*. Zhang et al[59] propose that CA inhibitors can block AQPs. In conclusion, CA and AQP1 inhibitors may serve as potential therapeutic options for antithrombosis without bleeding risk.

## 4.5 | A novel target for anti-inflammation

Procoagulant platelets also mediate inflammation by releasing PMPs and polyP, suggesting their potential as targets for anti-inflammation. PMPs and polyP also exert procoagulant effects; thus, they may also be potential targets for curing inflammatory and thrombotic disorders. A few studies have attempted to inhibit inflammation by inhibiting platelet function. However, annexin A5, a physiological anticoagulant protein, binds to and endocytoses PS, thereby inhibiting the release of PMPs.[60] This finding suggests that the human body has its own protective mechanism to resist thrombosis and inflammation, to a certain extent. Thus, the development of supplementary medicine based on this property is a promising research field but still has a long way to go.

### CONFLICT OF INTEREST
The authors declare that they have no competing interest.

### AUTHOR CONTRIBUTIONS
Yaxin Chu collected and summarized the literature. Han Guo and Yuncong Zhang structured and edited this review. Rui Qiao conceptualized this review and reviewed the final manuscript.

### DATA AVAILABILITY STATEMENT
All data used in this review are presented in the paper.

### ORCID
*Yaxin Chu* https://orcid.org/0000-0002-3946-1280
*Yuncong Zhang* https://orcid.org/0000-0002-3322-2895

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