

Mechanism underlying N-(3-oxo-dodecanoyl)-L-homoserine lactone mediated intracellular calcium mobilization in human platelets

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ABSTRACT

Acyl-homoserine lactones (AHLs), are the key autoinducer molecules that mediate *Pseudomonas aeruginosa* associated quorum sensing. *P. aeruginosa* produces two types of AHLs; N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C₁₂ HSL) and N-butryl-L-homoserine lactone (C4 HSL). AHLs are not only regulating the virulence gene of bacteria but also influence the host cell functions by interkingdom signaling. In this study, we explored the mechanism of AHLs induced calcium mobilization in human platelets. We found that 3-oxo-C₁₂ HSL but not C4 HSL induces intracellular calcium release. 3-oxo-C₁₂ HSL induced calcium mobilization was majorly contributed from the dense tubular system (DTS). Furthermore, 3-oxo-C₁₂ HSL also stimulates the store-operated Ca²⁺ entry (SOCE) in platelet. Intracellular calcium rise was significantly lowered in rotenone, and bafilomycin pre-treated platelets suggesting partial involvement of mitochondria and acidic vacuoles. The significant effect of 3-oxo-C₁₂ HSL on calcium mobilization can alter the platelet functions that might results in thrombotic disorders in individuals infected with *P. aeruginosa*.

1. Introduction

Platelets, also known as thrombocytes, are the second most abundant cells present in the blood. They are highly sensitive and respond quickly to any external stimuli. They play an important role in blood clot formation following blood vessel injury (hemostasis) and also participate in thrombotic disorders (thrombosis) [1]. The unwanted activation of platelets in blood circulation can cause pathological thrombus formation, which leads to myocardial infarction, atherosclerosis, and other cardiovascular complications [2]. Infection-induced platelet adhesion and aggregation also contribute to the development of thrombotic disorders [3].

Calcium is an essential secondary messenger which regulates a wide range of cell functions. In platelets, the rise in intracellular Ca²⁺ contributes to the regulation of different functions like activation, adhesion, actin reorganization, shape change, and degranulation [4]. The

elevation of intracellular Ca²⁺ is caused by the release from intracellular calcium reserves as well as the entry of Ca²⁺ from the extracellular environment through plasma membrane [5]. Two separate Ca²⁺ stores have been reported in platelets; first, the Dense Tubular System (DTS), an analogue of the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) and second lysosomal like acidic organelles [4,6–8]. In addition to these stores, mitochondria also play an important role in the maintenance of Ca²⁺ homeostasis and regulate various functions of different eukaryotic cells [9,10], including platelets [11–14]. It is widely established that stimulation of platelet surface receptors leads to the activation of phospholipase C (PLC) which further hydrolyzes the phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ binds to the IP₃ receptor present on the surface of intracellular stores and release intracellular calcium, while DAG helps in the entry of Ca²⁺ from extracellular compartment [5,15]. Prolong elevation of intracellular Ca²⁺

Abbreviations: AHL, acyl-homoserine lactone; 3-oxo-C₁₂ HSL, N-(3-oxo-dodecanoyl)-L-homoserine lactone; C4 HSL, N-butryl-L-homoserine lactone; PLC, phospholipase C; QS, quorum sensing; SOCE, Store-operated Ca²⁺ entry; PIP₂, phosphatidylinositol 4,5-bisphosphate; IP₃, inositol trisphosphate; DAG, diacylglycerol; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; DTS, dense tubular system

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concentration causes cellular damage [16]. In order to maintain the cytosolic calcium level, a calcium pump is present on endoplasmic reticulum surface, i.e., sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) which helps in restoration of cytosolic Ca^{2+} into ER lumen [17]. SERCA isoform SERCA2b (100 kDa), highly sensitive to thapsigargin, is expressed on DTS whereas thapsigargin insensitive SERCA isoform present on acidic organelles are known as SERCA3 (97 kDa) helps to maintain the cytosolic Ca^{2+} level in platelets [18–21].

In immuno-compromised individuals, *Pseudomonas aeruginosa* causes a wide range of acute and chronic infection, including wound infection, pulmonary infection, and development of sepsis condition [22]. Besides, *P. aeruginosa* also produces a wide variety of virulence factors such as exotoxins, proteases, hydrogen cyanide, and pigments, etc. Synthesis of these virulence molecules is commonly regulated by autoinducers known as Acyl Homo-serine Lactones (AHLs) [23]. *P. aeruginosa* mainly produces two types of these autoinducers viz. 3-oxododecanoyl homoserine lactone (3-oxo- C_{12} HSL) and N-butyryl homoserine lactone (C4 HSL) [23]. Recently, it has been documented that these AHL molecules also interact and influence the host immune responses by interkingdom signaling [24,25]. Moreover, it has been evident that 3-oxo- C_{12} HSL has immunomodulatory activity in murine peritoneal macrophages [26]. Besides, 3-oxo- C_{12} HSL inhibits vasoconstrictor tone in porcine coronary and pulmonary arteries, that might exert a regional hemodynamic effect to benefit the invading bacteria [27]. 3-oxo- C_{12} HSL enhances the neutrophil chemotaxis by the reorganization of the actin cytoskeleton and phosphorylation of PLC γ 1 and Rac1/Cdc42 [28,29] and disrupts the integrity of epithelial junction [30]. 3-oxo- C_{12} HSL also modulates the function of NIH3T3 fibroblast cells through intracellular calcium rise [31]. Our recent report demonstrated that 3-oxo- C_{12} HSL activates platelet through intracellular calcium-mediated ROS generation [32]. In this study, we explored the 3-oxo- C_{12} HSL-mediated calcium mobilization in human platelets and elucidated pathways and stores involved in intracellular calcium release.

2. Materials and methods

2.1. Reagents

Purified N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo- C_{12} HSL), N-butyryl-L-homoserine lactone (C4 HSL), DMSO, thapsigargin, ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), U73122, 2-Aminoethoxydiphenyl borate (2-APB), and Aspirin were purchased from Sigma-Aldrich. Rotenone and bafilomycin A1 are the product of Santacruz Biotechnology. Fluo-4 AM was procured from Thermo Fisher Scientific. All the reagents utilized in this study were of analytical grade, and Type 1 deionized water (18.2 M Ω cm, Millipore) was used for the preparation of solutions.

2.2. Preparation of platelet-rich plasma and washed platelets

Human venous blood was collected in citrate phosphate dextrose from healthy volunteers (drug-free and non-smokers) with their written informed consent as per the guidelines of Institutional Ethical Committee, Motilal Nehru National Institute of Technology Allahabad, India (Ref. No. IEC/16–17/017). Platelets were isolated by differential centrifugation method, as previously described [33]. Briefly, blood was centrifuged for 10 min at 180 \times g, and platelet-rich plasma (PRP) was collected in the fresh falcon tube and incubated with aspirin (1 mM) at 37 °C in a water bath for 15 min. After the addition of EDTA (5 mM), PRP was centrifuged at 600 g for 10 min. Cells were further washed in buffer A (20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM MgCl_2 , 0.36 mM NaH_2PO_4 and 1 mM EGTA, supplemented with 5 mM glucose). Platelets were resuspended in buffer B, which was the same as buffer A lacking EGTA. The final count was adjusted to 1–3 \times 10⁸ cells/

ml and mixed carefully to avoid activation.

2.3. Measurement of intracellular calcium

Washed platelets were incubated with 5 μM of Fluo-4 AM for 45–50 min at 37 °C in the dark. After incubation, the Fluo-4 AM loaded platelets were washed and resuspended in buffer B. Fluorescence intensity of Fluo-4 AM loaded platelets were recorded in a fluorescence spectrophotometer (Perkin Elmer model LS45). The samples were excited at $\lambda = 495$ nm, and emission was recorded at $\lambda = 525$ nm. The fluorescence intensity for the first 60 s was recorded to achieve the basal level, after 60 s 3-oxo- C_{12} HSL or C4 HSL was added to the samples in a different set of experiments. DMSO was used as vehicle control, and fluorescence intensity was further recorded for 300 s.

2.4. Measurement of SOCE

SOCE measurements were performed by the method as described elsewhere [34]. Briefly, platelet cells were incubated with 5 μM Fluo-4 AM for 45–50 min at 37 °C in the dark. After 60 s of baseline, 3-oxo- C_{12} HSL (100 μM), or DMSO as vehicle control were added in a different set of samples, and after 300 s 1 mM of CaCl_2 was added, and fluorescence intensity was recorded up to 600 s.

2.5. Statistical analysis

All the given data are represented as means \pm SEM of at least three independent experiments. The one-way ANOVA and student's *t*-test was used for data analysis. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. 3-oxo- C_{12} HSL but not C4 HSL induces platelet intracellular calcium

The platelet activation mainly depends on Ca^{2+} signaling, which plays a crucial role in many essential cellular processes, including secretion, aggregation, and shape changes [35,36]. In this study, we found that 3-oxo- C_{12} HSL induces intracellular calcium in a dose-dependent manner, and the maximum rise was obtained at 100 μM (Fig. 1 a, b). In contrast, another important QS molecule C4 HSL does not affect the intracellular calcium levels (Fig. 1c, d). To explore the origin of observed cytosolic calcium rise, we further incubated the platelets separately with EGTA (1 mM) or CaCl_2 (1 mM) for 10 min, followed by treatment with 3-oxo- C_{12} HSL (100 μM). DMSO was served as vehicle control. 3-oxo- C_{12} HSL induced Fluo-4 fluorescence intensity was higher in the presence of CaCl_2 than EGTA (Fig. 2 a, b). The result suggests that 3-oxo- C_{12} HSL induces both the release of intracellular Ca^{2+} and the entry of Ca^{2+} from extracellular space to the cytoplasm.

Role of the Dense Tubular System (DTS) in 3-oxo- C_{12} HSL induced intracellular calcium rise.

The two major calcium reserves in platelets, viz. dense tubular system (DTS) and acidic organelles, are categorized on the basis of expression of different isoforms of SERCA on their surface. SERCA 2ab is present on the dense tubular system (DTS), which is highly sensitive to thapsigargin, a specific inhibitor of SERCA. In order to understand the source of intracellular calcium rise induced by 3-oxo- C_{12} HSL, Fluo4-AM loaded platelets were preincubated with thapsigargin (1 μM) for 20 min and then stimulated with 3-oxo C_{12} HSL. Results showed that thapsigargin prevented 3-oxo- C_{12} HSL induced intracellular Ca^{2+} rise (Fig. 3 a, b) which suggests that 3-oxo- C_{12} HSL mobilizes calcium majorly from DTS store. Intracellular calcium release from DTS majorly follows the PLC-IP₃ pathway [4]. So, to investigate the involvement of PLC-IP₃ axis in 3-oxo C_{12} HSL-induced calcium rise, platelets were preincubated with a PLC- β inhibitor U73122 (10 μM) and IP₃ receptor antagonist 2-APB (100 μM) separately in Ca^{2+} free buffer, followed by

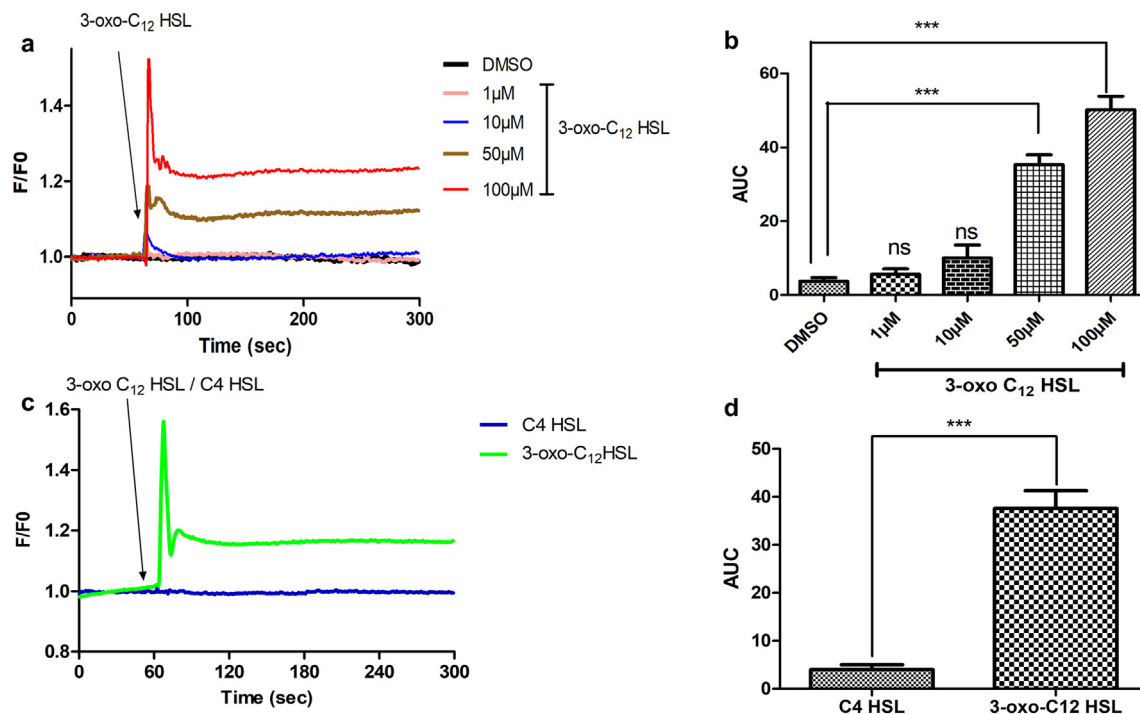


Fig. 1. 3-oxo-C₁₂ HSL but not C4 HSL evokes an increase in intracellular calcium in blood platelets. The blood platelets were loaded with Fluo-4 AM an intracellular calcium indicator that gives strong fluorescence when it is bind with calcium, and fluorescence intensity was measured in a fluorescence spectrophotometer. (a) The fluorescence intensity of Fluo-4 AM loaded Platelet was recorded for the first 60 s to achieve the baseline, and then stimulated with varying concentration of 3-oxo-C₁₂ HSL. (b) The bar graph shows an average of area under curve (AUC) of three independent experiments from (a). (c) The calcium traces show the platelet treated with 100 μM of either 3-oxo-C₁₂ HSL or C4 HSL. (d) The bar graph represents the average of AUC of three independent experiments from (c). In control experiments, 0.05% DMSO was used as vehicle control. F = fluorescence at 525 nm, F₀ = average fluorescence of first 60 s. Data represent Mean ± SEM of at least three independent experiments; ns = not significant, p*** < 0.001.

Ca²⁺ measurement. The results demonstrated that both the inhibitors significantly reduced the free intracellular calcium rise in the platelets (Fig. 3c, d). Altogether, these results indicate that 3-oxo-C₁₂ HSL increases the intracellular calcium levels by mobilization of calcium, majorly from DTS stores through PLC-IP₃ signaling cascade.

3.2. 3-oxo-C₁₂ HSL Stimulate SOCE in platelet

During platelet activation, the rise in intracellular Ca²⁺ is mediated mainly by two mechanisms: Ca²⁺ release from intracellular stores or by the entry of extracellular Ca²⁺ into the cytosol [37]. The decrease in ER calcium pool directly induces SOCE to refill the ER lumen through the entry of Ca²⁺ from extracellular space [38]. To investigate the effect of

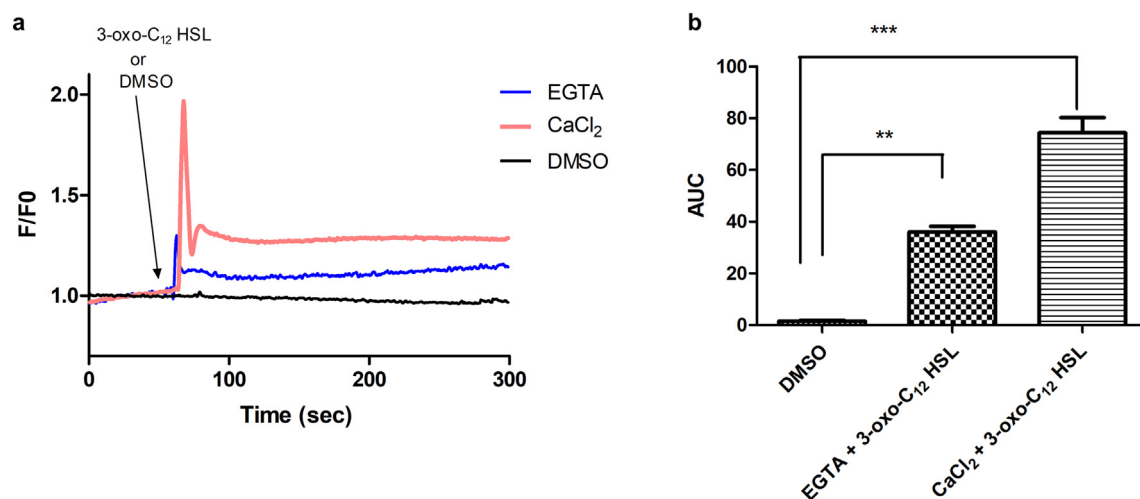


Fig. 2. 3-oxo-C₁₂ HSL raises free intracellular Ca²⁺ in platelet by intracellular calcium release and induces the entry of extracellular calcium to the cytoplasm. (a) Fluo-4 AM loaded Platelets were preincubated with either 1 mM CaCl₂ or 1 mM EGTA for 10 min, after achieving the baseline, 100 μM of 3-oxo-C₁₂ HSL was added in the sample, and fluorescence intensity was measured for further 300 s. (b) The bar graph represents the average of AUC of three independent experiments, from (a). In these experiments, 0.05% DMSO was used as vehicle control. Data represent Mean ± SEM of at least three independent experiments; p** < 0.01, p*** < 0.001.

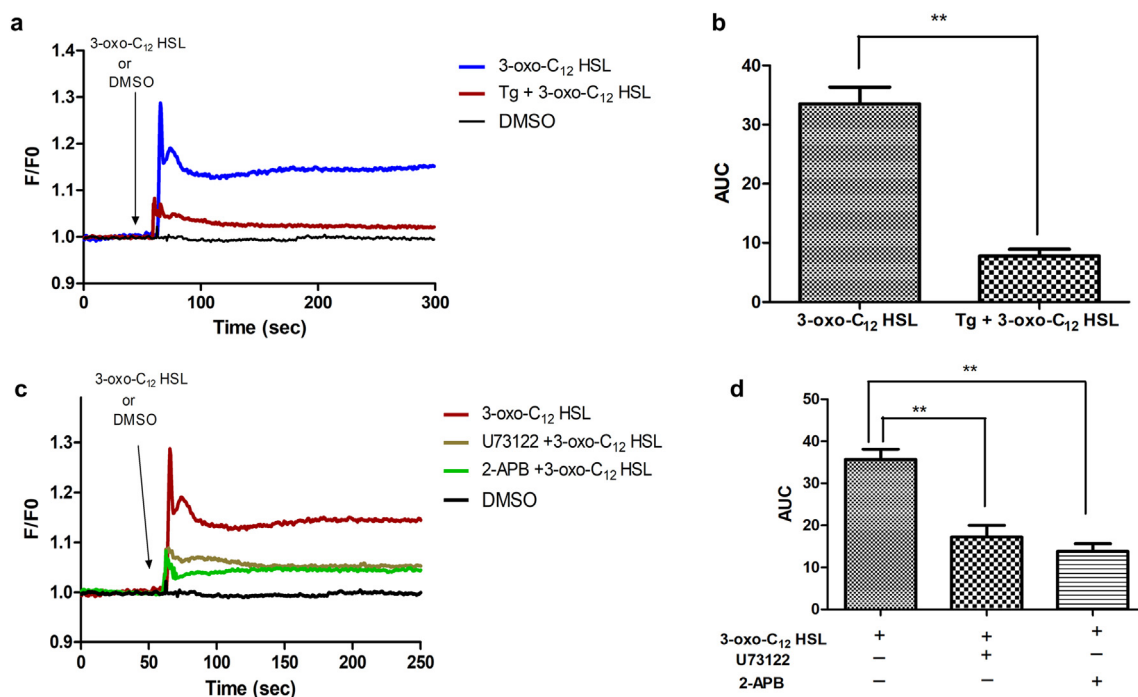


Fig. 3. 3-oxo-C₁₂ HSL induces calcium mobilization majorly from the DTS to the cytosol by PLC-IP3 axis in platelet.

(a) Fluo-4 AM loaded Platelets were pre-treated with 1 μ M of Thapsigargin (Tg) in the presence of 1 mM EGTA to deplete the DTS store, after achieving the baseline, 100 μ M of 3-oxo-C₁₂ HSL was added in the sample pre-treated with or without Tg, and fluorescence intensity was measured for further 300 s. (b) The bar graph represents the average of AUC of three independent experiments, from (a). (c) Fluo-4 AM loaded platelets incubated in EGTA were pre-treated with or without U73122 or 2-APB, followed by addition of 3-oxo-C₁₂ HSL (100 μ M). (d) The bar diagram represents the average of AUC of three independent experiments, from (c). In these experiments, 0.05% DMSO was used as vehicle control. Data represent Mean \pm SEM of at least three independent experiments; $p^{**} < 0.01$.

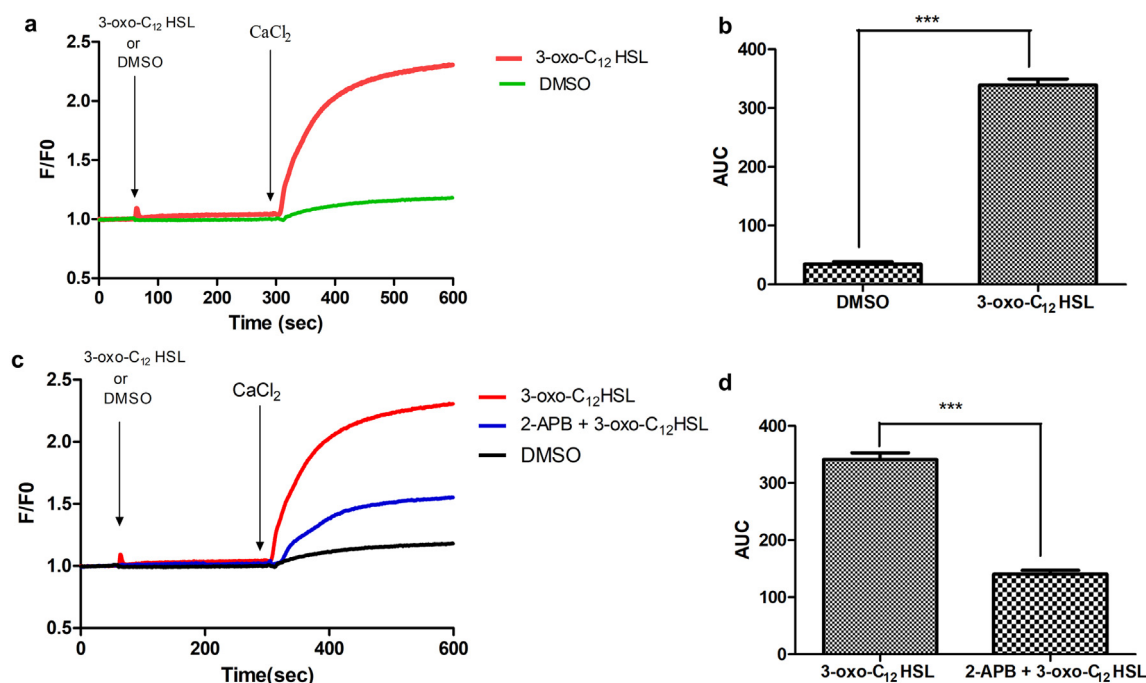


Fig. 4. 3-oxo-C₁₂ HSL stimulate Store-operated Ca²⁺ in platelet. (a) After achieving the baseline, 100 μ M of 3-oxo-C₁₂ HSL or DMSO vehicle control was added in Ca²⁺ free medium, 300 s later after addition of 3-oxo-C₁₂ HSL CaCl₂(1 mM) was added to the medium to initiate Ca²⁺ entry, and fluorescence intensity was measured up to 600 s. (b) The bar diagram represents the average of AUC of three independent experiments, from (a). (c) Fluo-4 AM loaded platelets were incubated with 2-APB (a potent SOCE blocker) for 10 min at 37 $^{\circ}$ C and then stimulated with 3-oxo-C₁₂ HSL (100 μ M) in Ca²⁺ free medium. After 300 s of the addition of 3-oxo-C₁₂ HSL, CaCl₂(1 mM) was added to the medium to initiate Ca²⁺ entry, and fluorescence intensity was measured up to 600 s. (d) The bar diagram represents the average of AUC of three independent experiments, from (c). Data represent Mean \pm SEM of at least three independent experiments; $p^{***} < 0.001$.

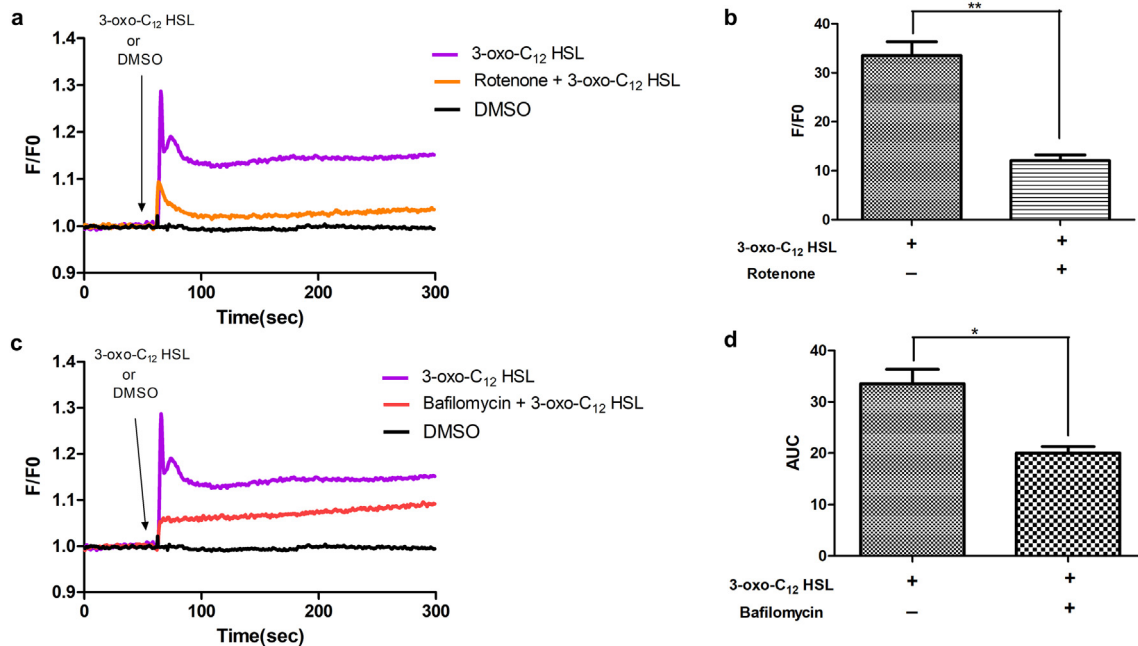


Fig. 5. Cytosolic calcium influx in 3-oxo-C₁₂ HSL treated platelet was partially contributed by both mitochondrial and acidic stores of calcium source. (a) Platelets incubated in EGTA were pre-treated with rotenone for 10 min, followed by addition of 3-oxo-C₁₂ HSL (100 μM), fluorescence was measured for 300 s. (b) The bar graph represents the average of AUC of three independent experiments, from (a). (c) Fluo-4 AM loaded platelets incubated in EGTA were pre-treated with bafilomycin A for 10 min, followed by addition of 3-oxo-C₁₂ HSL (100 μM), fluorescence was measured for 300 s. (d) The bar diagram represents the average of AUC of three independent experiments, from (c). In these experiments, 0.05% DMSO was used as vehicle control. Data represent Mean ± SEM of at least three independent experiments; p* < 0.05, p** < 0.01.

3-oxo-C₁₂ HSL on SOCE activation, the fluorescence of Fluo-4 AM loaded platelets were recorded, and after 1 min 3-oxo-C₁₂ HSL was added; subsequently, CaCl₂ (1 mM) was exposed to the extracellular medium. A prolonged elevation in intracellular calcium was observed in the presence of 3-oxo C₁₂ HSL indicating SOCE activation (Fig. 4 a, b). Further, 2-APB, a known SOCE blocker, significantly reduced the 3-oxo-C₁₂ HSL induced extracellular Ca²⁺ entry (Fig. 4 c, d). In these experiments, DMSO served as vehicle control. These findings indicate that 3-oxo-C₁₂ HSL stimulates the SOCE in platelets.

3-oxo-C₁₂ HSL induced Ca²⁺ rise is partially contributed by mitochondria and acidic organelles.

Next, we investigated the contribution of other intracellular calcium stores such as mitochondria and acidic vacuoles behind 3-oxo-C₁₂ HSL-induced intracellular calcium mobilization. To examine the possible role of mitochondria in 3-oxo-C₁₂ HSL induced calcium rise, platelets were pre-incubated with rotenone (20 μM), that disintegrates mitochondrial transmembrane potential and releases intracellular calcium from mitochondria. The result showed that 3-oxo-C₁₂ HSL induced calcium rise was significantly lowered in the presence of rotenone (Fig. 5 a, b). Furthermore, to inspect the involvement of acidic granules, intracellular calcium was measured in the presence of Bafilomycin A1 (1 μM), a potent and specific blocker of vacuolar-type H⁺-ATPase, that releases Ca²⁺ from acidic granules. It was found that 3-oxo-C₁₂ HSL induced calcium rise was diminished in the presence of Bafilomycin A1 (Fig. 5 c, d). These results suggest that mitochondria and acidic granules partially contribute to 3-oxo-C₁₂ HSL induced Ca²⁺ rise.

4. Discussion

The interaction between bacteria and host cells does not only depends upon the cell to cell contact but also on different toxins or diffusible QS molecules which regulates several virulence factors of bacteria, including biofilm formation. Although different families of QS molecules have now been described, the AHLs of gram-negative bacteria have been studied most extensively. These AHLs primarily differ in

length (from 4 to 18 carbon) and saturation level of their acyl chain having oxo or hydroxy substitutions at the C-3 position. *P. aeruginosa*, a gram-negative bacterium, mainly produces two types of AHLs, i.e., 3-oxododecanoyl homoserine lactone (3-oxo-C₁₂ HSL) and N-butyryl homoserine lactone (C4 HSL). Several studies have shown that the QS molecule 3-oxo-C₁₂ HSL modulates the function of different mammalian host cells by the phenomenon of interkingdom signaling [24,25]. Bacteria may enter to the circulatory system by indwelling medical devices, catheters, and other surgical processes [39] and it interacts with the blood cells, including platelets by direct or indirect mode of contacts and modulates their functions [40].

Recently, we have shown that *P. aeruginosa* quorum sensing molecule 3-oxo-C₁₂ HSL but not C4 HSL induces platelet activation by calcium-regulated ROS generation [32]. In this study, we have explored the pathway and stores involved behind 3-oxo-C₁₂ HSL mediated Ca²⁺ mobilization in platelets.

We found that 3-oxo-C₁₂ HSL induces intracellular calcium release in a dose-dependent manner, while C4 HSL did not show such activity. In platelets, the elevation in cytosolic calcium is either due to release from intracellular compartments or by the entry of Ca²⁺ from extracellular space through channels present on the plasma membrane. To understand the origin of 3-oxo-C₁₂ HSL induced intracellular Ca²⁺ rise in platelets, we performed the experiment in the presence of EGTA and CaCl₂ separately. It was observed that 3-oxo-C₁₂ HSL (100 μM) induced calcium elevation was higher in CaCl₂ than in the EGTA-treated sample. These results suggest that the 3-oxo-C₁₂ HSL induces both extracellular as well as intracellular calcium mobilization in platelets. In non-excitable cells, mobilization of Ca²⁺ in the cytoplasm is predominantly mediated by PLC-IP₃ pathway [15]. To confirm the involvement of PLC-IP₃ axis in 3-oxo-C₁₂ HSL induced calcium mobilization, the platelets were preincubated separately with U73122, an antagonist of PLCβ, and 2-APB, an IP₃ receptor antagonist. Treatment of these antagonists significantly reduced the calcium mobilization caused by 3-oxo-C₁₂ HSL. The activation of PLC hydrolyzes the PIP₂ into IP₃ and DAG [15]. IP₃ acts as a secondary messenger and binds to the IP₃ receptor and

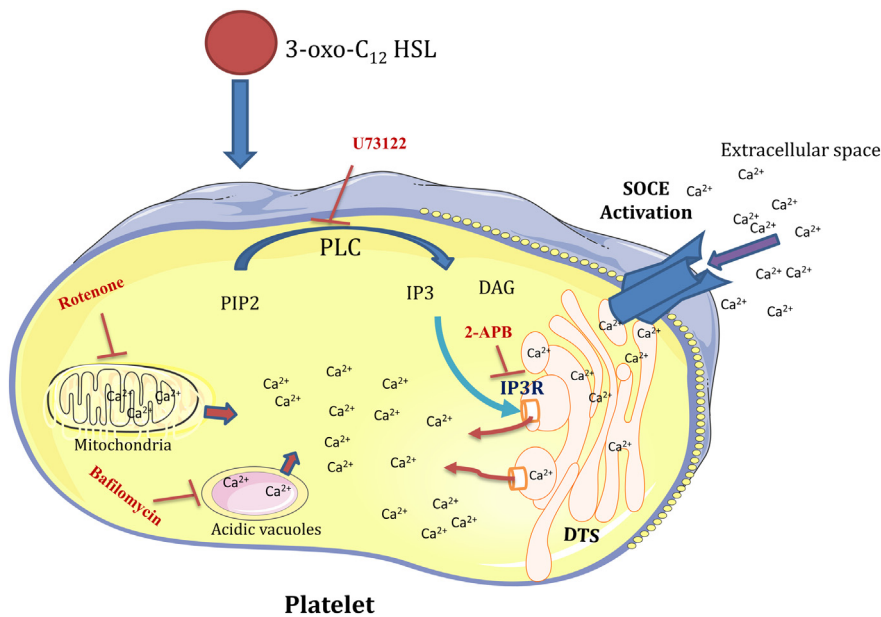


Fig. 6. A summary of 3-oxo-C₁₂ HSL induced calcium mobilization in platelets.

3-oxo-C₁₂ HSL activates the phospholipase C (PLC) which further hydrolyzes the phosphatidylinositol 4,5-bisphosphate (PIP₂) into Inositol trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ binds to the IP₃ receptor on Dense tubular system (DTS) and releases intracellular calcium into the cytoplasm. 3-oxo-C₁₂ HSL also potentiates the influx of calcium from extracellular space through the plasma membrane by SOCE activation. Furthermore, Mitochondria and acidic granules partially contribute to 3-oxo-C₁₂ HSL induced Ca²⁺ rise in platelets.

stimulate the Ca²⁺ release from DTS store to the cytoplasm [15]. Based on IP₃ binding and expression of two different SERCA isoforms (100 kDa and 97 kDa), two calcium stores are present in platelets [18]. Thapsigargin sensitive 100 kDa isoform of SERCA (SERCA2b) is found in the DTS membrane [18]. In order to assess the role of DTS store in 3-oxo-C₁₂ HSL induced Ca²⁺ mobilization, platelets were preincubated with thapsigargin for 20 min at 37 °C and then stimulated with 3-oxo-C₁₂ HSL (100 μM). Thapsigargin abrogated the 3-oxo-C₁₂ HSL induced Ca²⁺ rise, which confirms the significant contribution of DTS in 3-oxo-C₁₂ HSL stimulated Ca²⁺ mobilization. The depletion of intracellular calcium stores stimulate the entry of Ca²⁺ from extracellular space via plasma membrane channels and activate SOCE to refill these stores [41]. The SOCE activation mechanism is mainly regulated by the two protein families known as STIM and ORAI [42]. After depletion of the store, STIM moves to the DTS-plasma membrane and interacts with ORAI1 and thus induces calcium flow from extracellular compartments [42]. Since 3-oxo-C₁₂ HSL majorly induces the calcium rise through DTS stores, we further investigated whether 3-oxo-C₁₂ HSL is able to induce SOCE activation. For this purpose, the calcium influx in the Fluo-4 AM loaded platelets were monitored after the treatment with 3-oxo-C₁₂ HSL (100 μM) or DMSO and subsequent addition of CaCl₂ to the extracellular medium. The addition of CaCl₂ in the 3-oxo-C₁₂ HSL treated sample evokes a strong calcium influx compared to DMSO, suggesting the SOCE activation. Further, 2-APB, a well-known SOCE blocker, significantly reduced the calcium influx induced by 3-oxo-C₁₂ HSL. Thus, results suggest that 3-oxo-C₁₂ HSL depletes DTS calcium stores, which further stimulates the SOCE in the platelets. In order to examine the involvement of other probable calcium sources such as mitochondria and acidic stores in 3-oxo-C₁₂ HSL induced calcium mobilization, platelets were pretreated separately with rotenone and bafilomycin A1. The calcium mobilization induced by 3-oxo-C₁₂ HSL was significantly reduced in the presence of rotenone or bafilomycin A1, suggesting the partial contribution of the mitochondria and acidic vacuoles in 3-oxo-C₁₂ HSL induced calcium mobilization.

To conclude, our data indicate that 3-oxo-C₁₂ HSL mobilizes the intracellular calcium from DTS through PLC-IP₃ axis. Additionally, 3-oxo-C₁₂ HSL also potentiates the influx of extracellular calcium through the plasma membrane by SOCE activation. Mitochondria and acidic granules partially contribute to 3-oxo-C₁₂ HSL induced Ca²⁺ rise. A graphical summary of the current findings is represented in Fig. 6. These findings suggest that 3-oxo-C₁₂ HSL induced intracellular calcium mobilization can alter platelet functions. Thus, this study may further

be utilized for the development of new therapeutic drugs intended to reduce cardiovascular complications in *P. aeruginosa* infected individuals.

Author contributions

V.K.Y, V.A, and S.K.S conceived and designed the experiments; V.K.Y and P.K.S performed the experiments and analyzed the data; S.K.S and D.S provided comments and technical support; V.K.Y, S.K.S, and V.A wrote the paper.

Declaration of Competing Interest

No competing interest exists among the authors.

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References

- [1] Y. Wang, M. Andrews, Y. Yang, S. Lang, J. W. Jin, A. Cameron-Vendrig, G. Zhu, A. Rehem, H. Ni, Platelets in thrombosis and hemostasis: old topic with new mechanisms, *Cardiovasc. Hematol. Disord. Targets* 12 (2012) 126–132, <https://doi.org/10.2174/1871529X11202020126>.
- [2] M. Levi, T. Van Der Poll, M. Schultz, Infection and inflammation as risk factors for thrombosis and atherosclerosis, *Semin. Thromb. Hemost.* 38 (2012) 506–514, <https://doi.org/10.1055/s-0032-1305782>.
- [3] A. Dewitte, S. Lepreux, J. Villeneuve, C. Rigotherier, C. Combe, A. Ouattara, J. Ripoche, Blood platelets and sepsis pathophysiology: a new therapeutic prospect in critical ill patients? *Ann. Intensive Care* 7 (2017) 115, <https://doi.org/10.1186/s13613-017-0337-7>.
- [4] D. Varga-Szabo, A. Braun, B. Nieswandt, Calcium signaling in platelets, *J. Thromb. Haemost.* (2009) 1057–1066, <https://doi.org/10.1111/j.1538-7836.2009.03455.x>.

- [5] M.J. Berridge, M.D. Bootman, H.L. Roderick, Calcium signalling: dynamics, homeostasis and remodelling, *Nat. Rev. Mol. Cell Biol.* 4 (2003) 517–529, <https://doi.org/10.1038/nrm1155>.
- [6] L. Cavallini, M. Coassin, A. Alexandre, Two classes of agonist-sensitive Ca^{2+} stores in platelets, as identified by their differential sensitivity to 2,5-di-(tert-butyl)-1,4-benzohydroquinone and thapsigargin, *Biochem. J.* 310 (1995) 449–452, <https://doi.org/10.1042/bj3100449>.
- [7] E. Mammadova-Bach, M. Nagy, J.W.M. Heemskerk, B. Nieswandt, A. Braun, Store-operated calcium entry in thrombosis and thrombo-inflammation, *Cell Calcium* 77 (2019) 39–48, <https://doi.org/10.1016/j.ceca.2018.11.005>.
- [8] J.J. López, C. Camello-Almaraz, J.A. Pariente, G.M. Salido, J.A. Rosado, Ca^{2+} accumulation into acidic organelles mediated by Ca^{2+} - and vacuolar H^{+} -ATPases in human platelets, *Biochem. J.* 390 (2005) 243–252, <https://doi.org/10.1042/bj20050168>.
- [9] C. Villalobos, L. Nuñez, M. Montero, A.G. García, M.T. Alonso, P. Chamero, J. Alvarez, J. García-Sancho, Redistribution of Ca^{2+} among cytosol and organelle during stimulation of bovine chromaffin cells, *FASEB J.* 16 (2002) 343–353, <https://doi.org/10.1096/fj.01-0630com>.
- [10] T.J. Collins, M.J. Berridge, P. Lipp, M.D. Bootman, Mitochondria are morphologically and functionally heterogeneous within cells, *EMBO J.* 21 (2002) 1616–1627, <https://doi.org/10.1093/emboj/21.7.1616>.
- [11] S.I. Obyednyy, A.N. Sveshnikova, F.I. Ataullakhanov, M.A. Pantelev, Dynamics of calcium spiking, mitochondrial collapse and phosphatidylserine exposure in platelet subpopulations during activation, *J. Thromb. Haemost.* 14 (2016) 1867–1881, <https://doi.org/10.1111/jth.13395>.
- [12] H.J. Choo, T.B. Saafir, L. Mkumba, M.B. Wagner, S.M. Jobe, Mitochondrial calcium and reactive oxygen species regulate agonist-initiated platelet phosphatidylserine exposure, *Arterioscler. Thromb. Vasc. Biol.* 32 (2012) 2946–2955, <https://doi.org/10.1161/ATVBAHA.112.300433>.
- [13] A. Kholmukhamedov, R. Janecke, H.-J. Choo, S.M. Jobe, The mitochondrial calcium uniporter regulates procoagulant platelet formation, *J. Thromb. Haemost.* 16 (2018) 2315–2321, <https://doi.org/10.1111/jth.14284>.
- [14] S. Kumari, D. Dash, Melatonin elevates intracellular free calcium in human platelets by inositol 1,4,5-trisphosphate independent mechanism, *FEBS Lett.* 585 (2011) 2345–2351, <https://doi.org/10.1016/j.febslet.2011.05.067>.
- [15] G. Bird, O. Aziz, J.-P. Lievreumont, B. Wedel, M. Trebak, G. Vazquez, J. Putney Jr, Mechanisms of phospholipase C-regulated calcium entry, *Curr. Mol. Med.* 4 (2004) 291–301, <https://doi.org/10.2174/1566524043360681>.
- [16] B. Zhivotovsky, S. Orrenius, Calcium and cell death mechanisms: a perspective from the cell death community, *Cell Calcium* 50 (2011) 211–221, <https://doi.org/10.1016/j.ceca.2011.03.003>.
- [17] F. Wuytack, L. Raeymaekers, L. Missiaen, Molecular physiology of the SERCA and SPCA pumps, *Cell Calcium* 32 (2002) 279–305, <https://doi.org/10.1016/S0143416002001847>.
- [18] J. Enouf, R. Bredoux, B. Papp, I. Djaffar, A.M. Lompré, N. Kieffer, O. Gayet, K. Clemetson, F. Wuytack, J.P. Rosa, Human platelets express the SERCA 2-b isoform of Ca^{2+} -transport ATPase, *Biochem. J.* 286 (1992) 135–140, <https://doi.org/10.1042/bj2860135>.
- [19] R. Bobe, R. Bredoux, F. Wuytack, R. Quarck, T. Kovacs, B. Papp, E. Corvazier, C. Magnier, J. Enouf, The rat platelet 97-kDa Ca^{2+} ATPase isoform is the sarcoendoplasmic reticulum Ca^{2+} ATPase 3 protein, *J. Biol. Chem.* 269 (1994) 1417–1424.
- [20] F. Wuytack, B. Papp, H. Verboomen, L. Raeymaekers, L. Dode, R. Bobe, J. Enouf, S. Bokkala, K.S. Authi, R. Casteels, A sarco/endoplasmic reticulum Ca^{2+} -ATPase 3-type Ca^{2+} pump is expressed in platelets, in lymphoid cells, and in mast cells, *J. Biol. Chem.* 269 (1994) 1410–1416.
- [21] R. Flaumenhaft, SERCA navigating calcium signaling in platelets, *Blood* 128 (2016) 1034–1035, <https://doi.org/10.1182/blood-2016-07-723965>.
- [22] S.L. Gellatly, R.E.W. Hancock, *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses, *Pathog. Dis.* 67 (2013) 159–173, <https://doi.org/10.1111/2049-632X.12033>.
- [23] C.M. Waters, B.L. Bassler, Quorum sensing: cell-to-cell communication in bacteria, *Annu. Rev. Cell Dev. Biol.* 21 (2005) 319–346, <https://doi.org/10.1146/annurev.cellbio.21.012704.131001>.
- [24] E.K. Shiner, K.P. Rumbaugh, S.C. Williams, Inter-kingdom signaling: deciphering the language of acyl homoserine lactones, *FEMS Microbiol. Rev.* 29 (2005) 935–947, <https://doi.org/10.1016/j.femsre.2005.03.001>.
- [25] M.M. Kendall, V. Sperandio, What a dinner party! mechanisms and functions of interkingdom signaling in host-pathogen associations, *MBio.* 7 (2016) e01748-15, <https://doi.org/10.1128/mBio.01748-15>.
- [26] Y. Glucksam-Galnoy, R. Sananes, N. Silberstein, P. Krief, V.V. Kravchenko, M.M. Meijler, T. Zor, The bacterial quorum-sensing signal molecule N-3-Oxo-Dodecanoyl-L-Homoserine lactone reciprocally modulates pro- and anti-inflammatory cytokines in activated macrophages, *J. Immunol.* 191 (2013) 337–344, <https://doi.org/10.4049/jimmunol.1300368>.
- [27] S.M. Gardiner, S.R. Chhabra, C. Harty, P. Williams, D.I. Pritchard, B.W. Bycroft, T. Bennett, Haemodynamic effects of the bacterial quorum sensing signal molecule, N-(3-oxododecanoyl)-L-homoserine lactone, in conscious, normal and endotoxaemic rats, *Br. J. Pharmacol.* 133 (2001) 1047–1054, <https://doi.org/10.1038/sj.bjp.0704174>.
- [28] T. Karlsson, F. Musse, K.-E. Magnusson, E. Vikström, N-Acylhomoserine lactones are potent neutrophil chemoattractants that act via calcium mobilization and actin remodeling, *J. Leukoc. Biol.* 91 (2012) 15–26, <https://doi.org/10.1189/jlb.0111034>.
- [29] S. Zimmermann, C. Wagner, W. Müller, G. Brenner-Weiss, F. Hug, B. Prior, U. Obst, G.M. Hänsch, Induction of neutrophil chemotaxis by the quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone, *Infect. Immun.* 74 (2006) 5687–5692, <https://doi.org/10.1128/IAI.01940-05>.
- [30] E. Vikström, L. Bui, P. Konradsson, K.E. Magnusson, Role of calcium signalling and phosphorylations in disruption of the epithelial junctions by *Pseudomonas aeruginosa* quorum sensing molecule, *Eur. J. Cell Biol.* 89 (2010) 584–597, <https://doi.org/10.1016/j.ejcb.2010.03.002>.
- [31] E.K. Shiner, D. Terentyev, A. Bryan, S. Sennoune, R. Martinez-zaguilan, G. Li, S. Gyorke, S.C. Williams, K.P. Rumbaugh, *Pseudomonas aeruginosa* autoinducer modulates host cell responses through calcium signalling, *Cell. Microbiol.* 8 (2006) 1601–1610, <https://doi.org/10.1111/j.1462-5822.2006.00734.x>.
- [32] V.K. Yadav, P.K. Singh, M. Kalia, D. Sharma, S.K. Singh, V. Agarwal, *Pseudomonas aeruginosa* quorum sensing molecule N-3-oxo-dodecanoyl-L-homoserine lactone activates human platelets through intracellular calcium-mediated ROS generation, *Int. J. Med. Microbiol.* 308 (2018) 858–864, <https://doi.org/10.1016/j.ijmm.2018.07.009>.
- [33] S.K. Singh, M.K. Singh, P.P. Kulkarni, V.K. Sonkar, J.J.A. Grácio, D. Dash, Amine-modified graphene: Thrombo-protective safer alternative to graphene oxide for biomedical applications, *ACS Nano* 6 (2012) 2731–2740, <https://doi.org/10.1021/nn300172t>.
- [34] J. Prudent, N. Popgeorgiev, R. Gadet, M. Deygas, R. Rimokh, G. Gillet, Mitochondrial Ca^{2+} uptake controls actin cytoskeleton dynamics during cell migration, *Sci. Rep.* 6 (2016) 36570, <https://doi.org/10.1038/srep36570>.
- [35] W.S. Nesbitt, S. Giuliano, S. Kulkarni, S.M. Dopheide, I.S. Harper, S.P. Jackson, Intercellular calcium communication regulates platelet aggregation and thrombus growth, *J. Cell Biol.* 160 (2003) 1151–1161, <https://doi.org/10.1083/jcb.200207119>.
- [36] S.P. Jackson, W.S. Nesbitt, S. Kulkarni, Signaling events underlying thrombus formation, *J. Thromb. Haemost.* 1 (2003) 1602–1612, <https://doi.org/10.1046/j.1538-7836.2003.00267.x>.
- [37] T.J. Rink, S.O. Sage, Calcium signaling in human platelets, *Annu. Rev. Physiol.* 52 (1990) 431–449, <https://doi.org/10.1146/annurev.ph.52.030190.002243>.
- [38] M. Prakriya, R.S. Lewis, Store-operated calcium channels, *Physiol. Rev.* 95 (2015) 1383–1436, <https://doi.org/10.1152/physrev.00020.2014>.
- [39] S.L. Percival, L. Suleman, C. Vuotto, G. Donelli, Healthcare-associated infections, medical devices and biofilms: risk, tolerance and control, *J. Med. Microbiol.* 64 (2015) 323–334, <https://doi.org/10.1099/jmm.0.000032>.
- [40] J.R. Fitzgerald, T.J. Foster, D. Cox, The interaction of bacterial pathogens with platelets, *Nat. Rev. Microbiol.* (2006), <https://doi.org/10.1038/nrmicro1425>.
- [41] R.S. Lewis, The molecular choreography of a store-operated calcium channel, *Nature* 446 (2007) 284–287, <https://doi.org/10.1038/nature05637>.
- [42] D. Varga-Szabo, A. Braun, B. Nieswandt, STIM and Orai in platelet function, *Cell Calcium* 50 (2011) 270–278, <https://doi.org/10.1016/j.ceca.2011.04.002>.