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EXECUTIVE SUMMARY OF THE THESIS

Modeling thrombosis in a microfluidic system: the effect of shear stress, microparticles and protein surfaces

TESI MAGISTRALE IN BIOMEDICAL ENGINEERING – INGEGNERIA BIOMEDICA

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1. Introduction

Cardiovascular diseases are a group of heart and blood vessels disorders representing the leading cause of mortality and morbidity worldwide.
After a vessel wall injury the hemostatic process activates, and platelets allow the formation of a plug to stop the blood loss. This process is tightly regulated to allow a localized action, achieved by the balance between the procoagulant and anticoagulant systems. In case of platelet dysfunctions, this balance is not ensured, with the risk of either excessive bleeding or thrombosis [1]. Thrombosis, in particular, is the pathological clot formation within blood vessels.
Platelet dysfunctions are the major complications of cardiovascular diseases; they can also lead to severe consequences, such as ischemic stroke and myocardial infarction. Platelet disorders are often associated with high levels in blood of platelet-derived microparticles (PDMPs) [2]. They are subcellular particles, with a size of 0.1-1 μm, deriving from platelet membrane blebs. PDMPs are released upon activation, apoptosis, cardiovascular diseases, inflammation, and they can be involved in pathological processes such as infection transfer, tumorigenesis and thrombosis.
The standard treatment for platelet disorders is antiplatelet therapy, however, it does not consider the individuality of patients' response to antiplatelet agents. In some cases, despite antiaggregant administration, thrombosis or thromboembolism can still occur, as in the case of COVID-19, an acute respiratory syndrome that can cause hypercoagulability [3]. In view of the many risks associated to platelet dysfunctions, there is the impelling need to find new and improved methods to monitor platelet activity and identify tailored therapies.

1.1. Aim of the work

The present work explores the mechanisms behind thrombus formation by means of a microfluidic platform. The aim of the work is twofold:

- The characterization of the microfluidic platform for the assessment of platelet activation as a function of shear stresses and protein substrates, to also evaluate the effect of PDMPs. This was carried out by a joint collaboration between Politecnico di Milano and the University of Arizona.

- A refinement of the platform for the design of a label-free device to assess thrombus formation by monitoring the pressure inside the channel of a microfluidic device. This system was used to evaluate the effect of COVID-19 on platelet activity. This was conducted in the framework of an ongoing collaboration between Politecnico di Milano and Università degli studi di Milano (San Paolo Hospital campus).

2. State of the art

Platelet disorders or defects, if not promptly diagnosticated, may lead to severe complications. Platelet function testing (PFT) in the clinical setting has become essential for the assessment of platelet dysfunctions and to evaluate patients' response to antiplatelet therapy; prospects see the use of PFT also in general laboratories. Many PFT methods are available, and work according to different methodological principles, such as viscoelastic methods, tests based on aggregation and tests based on shear-mediated platelet activation [4]. Some of the most common devices to test platelet activity are PFA-100, VerifyNow System, TEG-5000, Multiplate Electrode Aggregometry; these devices are easy-to-use, and they mainly use whole blood (WB), which eliminates the necessity of processing the blood samples. However, most of PFT devices are expensive, time consuming, poorly standardized and do not evaluate thrombus formation under physiological flow conditions. Flow assays are indispensable research tools that allow to replicate the vasculature hemodynamic conditions. Over the last decade, microfluidic systems extensively developed. Microscale systems allow a precise control of the confined fluid behavior and enable the investigation of phenomena non-visible at the macroscopic level. Microfluidic flow assays allow a good control of the flow inside the microchannels. They can potentially be used in clinical applications to diagnose and treat platelet disorders like thrombosis or excessive bleeding. Scavone et al. [5] investigate the shear-mediated platelet activity and the influence of assay-dependent variables using polydimethylsiloxane (PDMS) microfluidic devices with collagen-coated microchannels, which allow to simulate the presence of a vessel wall injury. Thrombus formation inside the microfluidic devices is visualized using fluorescence microscopy; the number of thrombi and their morphological features are analyzed.

An alternative method, proposed by Hosokawa et al. [6], uses a flow pressure sensor to evaluate thrombus formation by monitoring the pressure drop in the microfluidic channel.

Another important diagnostic element to detect several diseases, such as stroke, thrombo-embolisms, myocardial infarction, sepsis, is the monitoring of PDMP levels in blood. Immunofluorometric assays, flow cytometry, spectroscopy, dynamic light scattering are methods used to detect and analyze PDMPs [2].

In pathological conditions, especially when an inflammatory component is involved, their concentration in blood significantly increase, hence, PDMP count in blood is used as a marker for several diseases. High PDMP levels were also found in COVID-19 patients, where platelet activity was altered: despite the higher platelet activation, platelets were reported to be less responsive to agonist stimulation [7].

3. Materials and methods

3.1. Microfluidic platform

The effects of flow on blood cell adhesion and thrombus formation are studied, ex vivo, using a microfluidic platform (Figure 1a). It consists of a syringe pump, a fluorescence microscope and a PDMS chip, featuring 6 parallel channels with rectangular cross section, bonded on a microscope slide (Figure 1b).

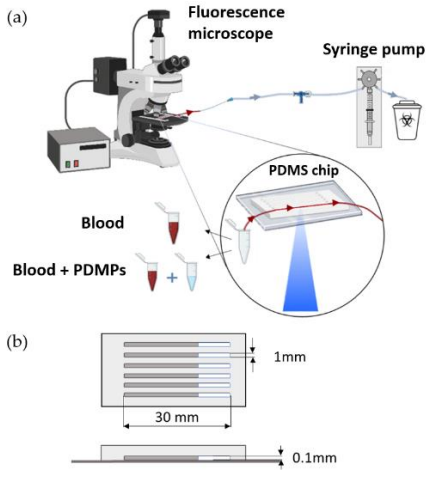

Figure 1. (a) Experimental set-up; (b) PDMS chip.

The capillaries are coated, for 1/3 of their length starting from the end, with extracellular matrix proteins at a concentration of 100 μg/mL: fibrillar collagen type I, fibrinogen from human plasma, purified laminin, pure fibronectin, recombinant vitronectin.

Hirudin-anticoagulated WB (recombinant hirudin, 450 ATU/mL blood) is perfused, for four minutes, into the coated channels under various experimental conditions. The effect of the different adhesive proteins on platelet adhesion and aggregation was evaluated (n=7).
Using a fluorescence microscope (Nikon ECLIPSE Ni) coupled to a 14-bit CCD camera (Nikon DS-Qi2), images were taken at the end of the test and a MATLAB code computed four parameters: surface coverage (SC [%]), the percentage of the area covered by platelets; number of thrombi (Nth); mean thrombus area (Ath) [μm²], defined as the SC divided by the Nth; mean fluorescence intensity (FI). The influence of the shear stress was investigated. Three shear stresses were tested, representing typical values in veins, large arteries and arterioles (13.5, 49.5 and 72 dyne/cm², respectively).
The addition of PDMPs to the blood sample allows to test their effect on thrombus formation on a collagen (100 μg/mL) coated surface, at the above-mentioned shear stress levels. WB and WB+PDMPs were perfused; results from the two groups were analyzed and compared (n=7).
To start developing a label-free device to measure platelet activity, a differential pressure sensor (PendoTECH™ PRESS-S-000) was introduced in the platform, placed at the channel outlet. This allows to monitor and record the pressure change in the microchannel due to thrombus formation. A MATLAB code processes the sensor output signal, providing the pressure measurements and a pressure-time curve. The pressure drop caused by minor head losses (ΔP) due to thrombus formation, is also evaluated. Experiments were conducted at arterial and arteriolar shear stress levels (n=12), using two different collagen concentrations (10 and 100 μg/mL), with the aim of finding a correlation between ΔP and thrombus formation. A venous shear stress was not tested, as it leads to too small pressure variations to be appreciated by the used sensor. The thrombus formation in the channel is expressed by means of both FI and channel occlusion rate. The latter is evaluated from thrombus volume measurements, obtained by confocal microscopy (Leica DM IRE2). For this part of the work, the analyzed blood samples were withdrawn from healthy subjects (n=6) and covid patients (n=6). Therefore, results were divided in these two groups and compared, to evaluate the presence of any difference in platelet activity by means of FI, ΔP and channel occlusion rate.

3.2. PDMPs generation

The effect of high PDMP concentrations in blood has been assessed by generating and introducing microparticles in the blood sample.
PDMPs were obtained adding calcium chloride (2.5 mM CaCl2) to 350 μL of gel-filtrated platelets (100000 platelets/μL), sonicating the solution (50% of power, 10s, Branson Ultrasonics™ SLPt Sonifier) and centrifuging it twice at 2000rpm for 15 minutes to sediment platelets and obtain PDMP fraction. Then the supernatant containing PDMPs was collected, aliquoted and frozen at -80°C. Prior to the experiments, PDMPs were thawed and added to the WB sample at a ratio blood:PDMPs of 10:1.

4. Results

4.1. Effect of immobilized proteins

Experiments under flow were performed in a microfluidic system and platelet adhesion was investigated on all protein substrates at a same shear stress level using blood from a same donor. Results are shown in Figure 2.
SC is the main indicator for platelet adhesion (Figure 2a). On collagen and fibrinogen substrates good platelet adhesion is visible at every shear stress level (SC [%] ± SD = 11.4±4.5, 7.5±4.4 and 25.3±10.4 for collagen; 12.4±3.1, 10.4±4.6 and 9.3±3.1 for fibrinogen, at 13.5, 49.5 and at 72 dyne/cm², respectively). SC results highlight almost no adhesion on vitronectin at all shear stress levels (SC [%] ± SD = 0.05±0.05 at 72 dyne/cm²; $p<0.0001$ compared to the collagen substrate). Low adhesion is present on fibronectin (SC [%] ± SD = 0.4±0.3 at 72 dyne/cm²). On laminin, good adhesion is achieved at low shear stress, but it drastically reduces at high shear stress (SC [%] ± SD = 6.3±3.3 and 0.4±0.3 at 13.5 and 72 dyne/cm², respectively). Ath and FI (Figure 2c and d) mainly account for platelet aggregation. Results show a significant increase of Ath and FI, with increasing shear stress, only for the collagen substrate (Ath [μm²] ± SD = 69±17 and 514±194 at 13.5 and 72 dyne/cm², respectively. FI ± SD = 2467±754 and 6211±1957 at 13.5 and 72 dyne/cm², respectively). At the highest shear stress level, significant differences in Ath and FI are present between collagen and all the other substrates ($p<0.01$), suggesting that only collagen is involved in high shear-induced aggregation.

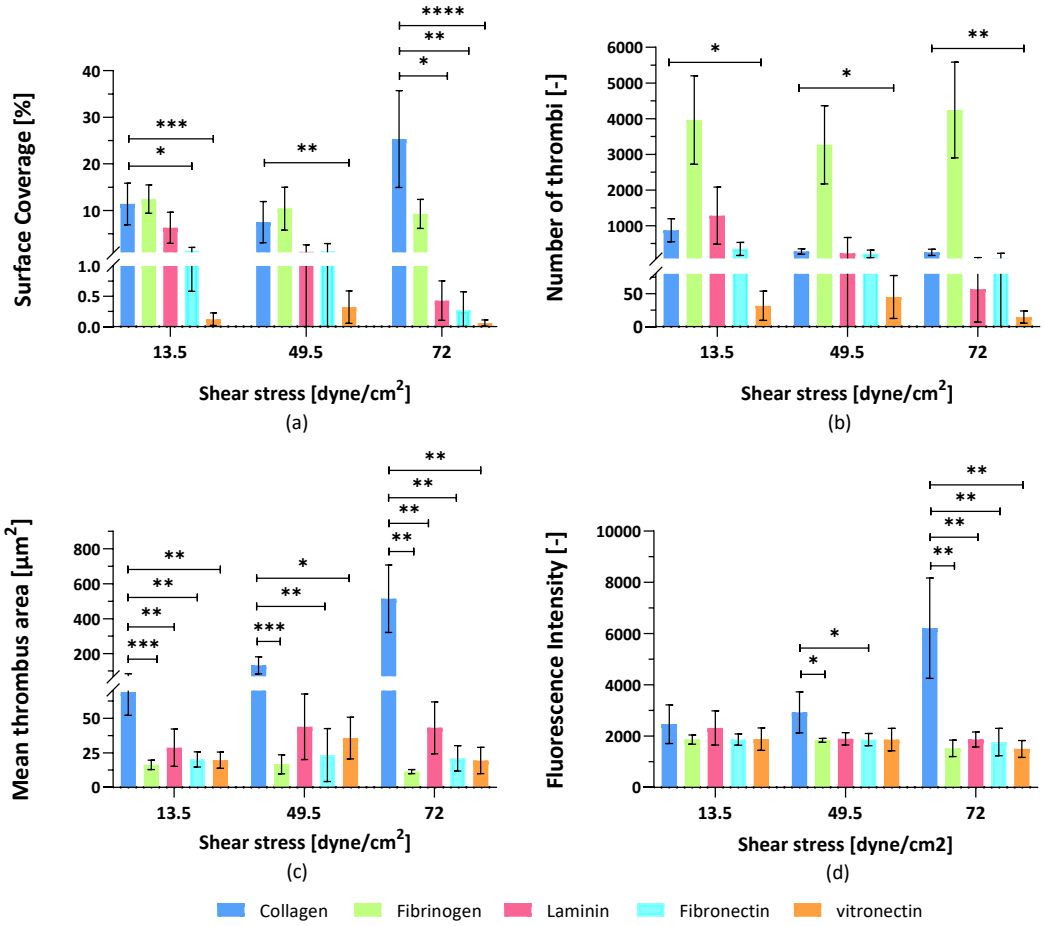

Figure 2. Results of shear induced adhesion and aggregation in whole blood on five different proteins substrates organized by shear stress value: (a) SC, (b) Nth, (c) Ath, (d) FI. Data from 7 donors are shown as mean ± SD. Normally distributed data were analyzed by one-way ANOVA, non-normally distributed data by Friedman test. *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001.

4.2. Effect of PDMPs

The effect of circulating PDMPs on platelet adhesion and aggregation on a collagen surface was assessed. The addition of PDMPs to WB leads to a reduction in size of the thrombi formed on the collagen substrate (Figure 3).

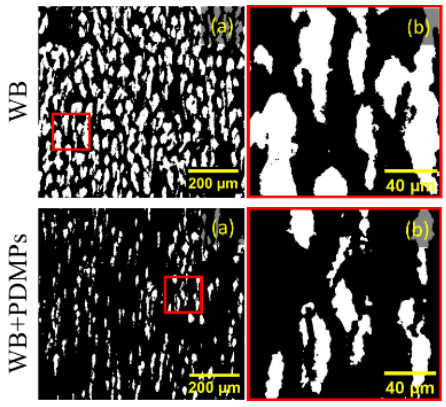

Figure 3. Effect of PDMPs on SC and Ath, at 72 dyne/cm² shear stress; thrombi in white. Top, no PDMPs are added; bottom, PDMPs are added to WB. (a) overall surface coverage; (b) magnified image of the thrombi (in white). Images captured by fluorescence microscope with 10X objective.

Data (Figure 4) confirm how, at all shear stress levels, but especially at the highest value, the addition of PDMPs leads to a reduced adhesion (SC [%] ± SD: 25.3±9.3 and 10.6±4.5 at shear value 72 dyne/cm² for WB and WB+PDMPs, respectively). High PDMP concentrations lead to an increase of the number of aggregates formed on the substrate, but their area and FI significantly reduce, suggesting a reduction of the platelet aggregation ($p<0.01$; Ath [µm²] ± SD: 522±186 and 196 ± 60 at shear value 72 dyne/cm² for WB and WB+PDMPs respectively; FI ± SD: 6482 ± 1486 and 3301 ± 888 at shear value 72 dyne/cm² for WB and WB+PDMPs respectively).

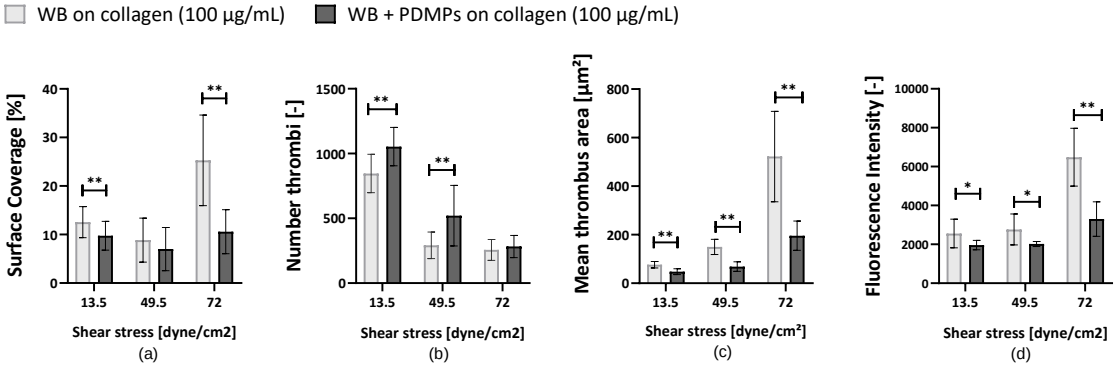

Figure 4. Effect of the addition of microparticles fraction on (a) SC, (b) Nth, (c) Ath and (d) FI on a collagen substrate. Data from 7 donors are shown as mean ± SD. Normally distributed data were analyzed by t-test, non-normally distributed data by Mann-Whitney test. *p <0.05, **p <0.01.

4.3. Pressure measurements

A new approach to evaluate platelet activity was tested. A pressure sensor was added to the platform and a validation of this set-up was performed. The pressure drop inside the channel, due to thrombus formation, was monitored and recorded. Pressure results were correlated with the FI and with the channel occlusion percentage due to thrombus formation inside the channel.
The pressure-fluorescence intensity dependence is characterized by a linear trend line (R^2=0.771) and showed a correlation coefficient (CC) equal to 0.88. On the other hand, the panel about the channel occlusion percentage is not well correlated with pressure measurements (R^2= 0.168 and CC= 0.41).

4.3.1. Effect of COVID-19

Using the refined platform the effect of COVID-19 on platelet activity, by means of FI, ΔP and channel occlusion rate, was assessed. Results show some changes in the evaluated parameters between the two groups (healthy subjects and covid patients); in particular, FI, ΔP and channel occlusion rate mean values are lower for the covid group. However, a statistical difference (p=0.035) was only found at arterial shear stress and low collagen concentration for the FI results. For both ΔP and channel occlusion rate, mean values between the two groups differ, but no relevant differences were highlighted at each test condition (p>0.05).

5. Discussion

The performed tests assess platelet affinity with the different immobilized adhesion proteins, trying to determine the contribution of each protein alone on thrombus formation, at different shear stresses. Collagen substrate is the only one allowing good platelet adhesion and aggregation at all the shear stress values. In fact, when exposed to blood flow, it binds von Willebrand factor (vWF) in plasma [8]. After the shear-mediated platelet activation, platelets bind to collagen through the platelet receptors GPVI and $\alpha 2\beta 1$; thanks to the high affinity between the collagen-bound vWF and the platelet receptors GPIb-V-IX and $\alpha IIb\beta 3$, platelet adhesion and aggregation is also granted at high shear stresses.
Fibrinogen elicits high platelet affinity, due to the $\alpha IIb\beta 3$ mediated platelet adhesion on this protein. Fibrinogen does not provide a significant contribution in platelet immobilization, in fact, only small thrombi, with a low Ath, formed on the substrate (Figure 2c).
Platelet adhesion to laminin depends, among others, on the integrin $\alpha 6\beta 1$. At low shear stresses, laminin elicits good platelet adhesion, comparable with the one on collagen and fibrinogen, but the formed aggregates present low Ath. At high shear stress adhesion becomes very low. The aggregates formed on fibronectin are also small and adhesion reduces with the shear stress (Figure 2a). Adhesion also depends on the typical association rate between platelet receptors and ligands. At high shear stresses the time of contact between platelets and protein is low, hence the adhesion reduces if the association rate is lower than platelet velocity.
Platelet adhesion on vitronectin is almost absent. In fact, in combination with other proteins vitronectin could contribute to the thrombus growth, but, alone, it allows almost no adhesion at all [9].

High PDMP levels are a marker of cardiovascular diseases or, more in general, of disorders associated with a pathological and increased platelet activity (e.g. COVID-19) that leads then to a greater PDMP formation [2]. The presence of microparticles attenuates the overall adhesion to the protein substrate and platelet-to-platelet aggregation, especially at high shear stresses: comparing the two sets of results (WB and WB+PDMPs), in fact, a reduction of the SC, hence platelet adhesion, and a reduction of Ath and FI, hence platelet aggregation, is noticeable (Figure 4). Many adhesion receptors are expressed on the surface of PDMPs [2], hence platelet stimulation promoted by PDMPs is higher than collagen-induced stimulation. Due to their high affinity, platelets in blood may adhere to circulating microparticles, rather than forming immobilized aggregates on the substrate, generating, possibly, activated microaggregates that flows in the bloodstream. In vivo, this scenario could lead to microvessel occlusion or even organ failure. This hypothesis is actually in accordance with findings on platelet activity in COVID-19 patients: COVID-19 is in fact associated with increased numbers of PDMPs, and many patients were reported to have increased levels of circulating activated platelet aggregates, with high surface levels of P-selectin [3].

The addition of the pressure sensor to the platform allowed to validate the pressure readings.
FI measurement is related to platelet aggregation and thrombus growth inside the channel. Therefore, the pressure drop caused by platelet aggregation was correlated with the FI results. The good correlation found confirms pressure ability to assess thrombus formation in the channel.
Thrombi formed inside the channel contribute to the minor head losses that cause the measured ΔP, however a bad correlation was found between pressure and channel occlusion rate.
This is mostly due to the poor resolution of the confocal microscope, since it has a limit in the maximum allowed height of the observed object (80μm): thrombus volume could not be measured at 100 μg/mL collagen concentration and 72 dyne/cm^2 shear stress, as the thrombi formed at this test condition are usually higher than this threshold. Therefore, not a very high number of samples were considered to determine the pressure-channel occlusion correlation, and this may have contributed to such a bad correlation.

Studies report changes in platelet activity for COVID-19 patients [3], so the effect of COVID-19 on thrombus formation was assessed. However, the only statistically significant difference was found for the FI at low collagen concentration and arterial shear stress. The small number of experiments performed for each donor group (n=6) may be the reason why no other relevant differences were highlighted. Therefore, more data are needed to confirm the absence or presence of any statistically significant difference.

6. Conclusions

The platform allows to determine the platelet adhesion and aggregation rate that characterizes each protein substrate, at each shear stress level. This leads to an important result: the effects of different events on platelet activity can be tested on the most suitable substrates for the phenomena that need to be investigated.

The effect of high levels of PDMPs was assessed by generating and introducing a controlled quantity of PDMPs in hirudin-anticoagulated WB. The obtained effect is a change in platelet activity, resulting in the reduction of the dimension of platelet aggregates formed on the collagen substrate. A hypothesis was made: flowing platelets adhere to circulating microparticles, determining the presence of circulating activated microaggregates that, in vivo, could cause microthrombosis or could lead, ultimately, to organ failure.

Since microscopy does not provide a rapid and real time assessment of thrombus formation, a first step towards the design of a label-free system for a rapid evaluation of platelet activity has been taken, and consisted in the pressure readings validation. Better volume measurements are needed to correlate pressure and channel occlusion rate and proceed with the device development, but the results achieved so far have the potential to build an easy-to-use, inexpensive, user-friendly device for the assessment of platelet function.

The effect of COVID-19 on platelet activity was assessed: small changes in platelet activity were found, but statistical analysis does not highlight relevant differences. More experiments should be performed, to further investigate the different platelet activity caused by COVID-19 and the relative pressure measurements.

7. Bibliography

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