PAMFix, a fixative developed to enable remote platelet function testing: uses and applications

Stan Heptinstall, Sue Fox, Jane May, Ann White and Natalia Dovlatova
Clinical Neuroscience, D-Floor, South Block, Queen’s Medical Centre, Nottingham, NG7 2UH, UK.

ABSTRACT

PAMFix is a fixative that was developed to stabilize the platelet biomarker P-selectin on activated platelets. Stabilization of this biomarker introduced the possibility of performing measurements of platelet function remotely where there was no access to special equipment or technical expertise. The idea was to use PAMFix to fix the platelets after stimulating platelets in blood, after which the stabilized samples would be transported to a central laboratory for analysis by flow cytometry. This would obviate the need for special equipment or technical expertise at or near the point at which the blood was collected and processed. Here we demonstrate the stability of samples of blood treated with PAMFix and describe several applications in which the use of PAMFix has proved to be particularly beneficial.

KEYWORDS: PAMFix, P-selectin, fixing solutions, remote platelet function testing, aspirin, clopidogrel, P2Y₁₂ antagonists

1. Introduction

There are many approaches to studying platelet function and many potential applications in relation to patient diagnosis, treatment and research [1, 2]. Different approaches to measurement of platelet function include measurement of platelet aggregation, the extent of release of the contents of platelet storage granules and the degree of expression of platelet activation markers on the platelet surface. All of these approaches have been used to quantify platelet function but they all require either special equipment or technical expertise for the measurements to be performed.

Platelet function measurements can yield information on the effectiveness of drugs known as antiplatelet agents that are used to reduce the contribution of platelets to thrombus formation in conditions such as heart attack and stroke. Measurements are also used in the development of new approaches to antiplatelet therapy, to exclude unwanted effects of drugs and foodstuffs, and also to identify platelet dysfunction which can be responsible for enhanced bruising and bleeding in some individuals. Platelet function measurements are also useful for applications in the veterinary field.

Platelet function is usually studied in freshly prepared whole blood or in platelet-rich plasma (PRP) derived from it. Studies are generally performed within an hour or two of obtaining the blood. Typically an agent that is known to activate platelets is added to the samples of blood or PRP and the consequences of this are measured in some way; for example, measurement of platelet aggregation in stirred samples of the blood or PRP. Another approach is to quantitate the level of expression of platelet activation markers (PAM) such as P-selectin on the surface of platelets consequent to translocation from α-granules within the platelets. The degree of platelet activation can then be measured by quantitating the amount of P-selectin using flow cytometry. However, the requirement for special equipment and/or special technical expertise at or near the point of blood withdrawal hampers the routine application of platelet function testing in clinical practice.
A means of overcoming this problem is to separate in time the immediate processing of a blood sample from the actual measurement that is performed. This can be achieved through the use of a fixative that stabilizes the treated blood sample such that it can then be transported to a central laboratory for the measurement to be performed.

This review focuses on the measurement of platelet function through quantitation of P-selectin, a biomarker that appears on the platelet surface following activation. This can be achieved through the use of PAMFix, a specially developed fixative which stabilizes the level of P-selectin on the platelet surface for at least 9 days (www.plateletsolutions.co.uk). PAMFix is now being incorporated into special platelet kits which can be used at the point of blood collection to prepare a blood sample for dispatch for remote analysis. This means that the only technical action needed at a time point close to blood collection is to activate platelets in blood followed by addition of PAMFix to stabilize the samples. PAMFix has also been used in kits to assess the effects of aspirin, P2Y12 antagonists and other antiplatelet agents on platelet function and also for assessment of platelet dysfunction. It is also being used in veterinary studies. In addition to providing stability for biomarkers of platelet aggregation such as P-selectin, PAMFix has also been proved useful in studies of platelet aggregation and von Willebrand factor binding to activated platelets, and may also find a use in the assessment of canine lymphoma.

### 2. Stability of P-selectin measurements using PAMFix

The data in figure 1 are results of studies performed at the University of Nottingham where the levels of P-selectin (CD-62P) on platelets in whole blood were determined after platelet stimulation in various ways and under various conditions so as to produce a wide range of levels of P-selectin. The blood was fixed using PAMFix and then stored for various times at either at room temperature or 4 °C prior to the P-selectin measurements. The level of P-selectin was determined using flow cytometry. Figure 1 also contains the results of a transportation study that was performed. It can be seen that sample stability was maintained for up to 9 days under all the conditions used.

More recently the time to flow cytometric analysis was extended following a study in which P-selectin samples were routinely analysed up to 3 days following fixation with PAMFix and then reanalysed at 28 days (Figure 2). PAMFix thus offers the opportunity to perform studies of platelet function in remote locations without the need for special facilities at the point where the blood is taken.

### 3. PAMFix for assessing inhibitory effects of aspirin and P2Y12 antagonists

A particular use for PAMFix is as a component of various platelet kits for measurements of platelet function, and several publications on the use of the platelet kits are now available [3-12].

The first kits produced were aspirin kits which were designed to measure platelet function in patients taking aspirin so as to assess the adequacy of the aspirin treatment in patients who receive this drug. P2Y12 kits were designed to determine the effects of P2Y12 antagonists such as clopidogrel, prasugrel, ticagrelor and cangrelor.

The approach used is to stimulate platelets in a sample of the patient’s blood with particular agents relevant to the drug under investigation, and then to stabilize the P-selectin on activated platelets using the PAMFix. The blood sample is then sent off to a central laboratory where the amount of the P-selectin on the platelet surface is measured using flow cytometry. Effective treatment with aspirin markedly reduces the amount of P-selectin that is exposed following platelet stimulation with arachidonic acid, and this forms the basis of the aspirin kit. Similarly, effective treatment with a P2Y12 antagonist markedly reduces the amount of P-selectin that is exposed following stimulation with adenosine diphosphate (ADP), and this forms the basis of the P2Y12 Kit.

### 3.1. Studies in healthy volunteers and patients with acute coronary syndromes

The first results based on this new approach were published by Fox et al. in 2009 [3]. Measurements were performed in blood from healthy volunteers and patients with acute coronary syndromes (ACS). It was found that aspirin added to blood in vitro profoundly inhibited P-selectin expression following platelet stimulation with arachidonic acid whereas the P2Y12 antagonist cangrelor had only a small
Patients with cardiovascular disease who exhibit high residual platelet function despite taking clopidogrel have a raised likelihood of a further thrombotic event. Consequently Thomas et al. [4] performed a study to determine whether high residual platelet function measured using P-selectin is also associated with further adverse cardiovascular events. This was a prospective study performed in patients with ACS who were receiving both clopidogrel and aspirin. The P-selectin measurements were carried out alongside light transmission aggregometry. The main results of the study are displayed in figure 4 where it can be seen that patients who experienced a further adverse event within 12 months of the index event displayed increased P-selectin, assessed using the P2Y12 test, compared with those patients who did not experience a further adverse event. The inhibitory effects of aspirin were substantial in both patient groups. The P2Y12 P-selectin test results correlated well with light transmission aggregometry (Spearman $r = 0.68$ for maximum and $r = 0.73$ for final aggregation, $p < 0.0001$). This study suggests that a P2Y12 P-selectin test is capable of detecting high on-treatment platelet reactivity, which is associated with recurrent cardiovascular events, in the same way as other approaches to measuring platelet function.

There are various platelet function testing methods available to monitor the effectiveness of antiplatelet agents in patients with cardiovascular disease and we therefore set out to formally compare our own tests with these existing procedures [5]. We performed this comparative study using blood samples from ACS patients treated with aspirin and clopidogrel ($n = 102$) or aspirin and prasugrel ($n = 56$) for at least one month. We compared the P-selectin testing kits with three other aspirin and four other P2Y12
Figure 3. P-selectin expression on platelets in blood from healthy volunteers (Groups A, B and C) and patients with acute coronary syndromes (Groups D and E) measured using the aspirin P-selectin test (left) or the P2Y12 P-selectin test (right). Results were obtained before (O) and after adding aspirin (+A) or cangrelor (+C) to the blood in vitro and also in volunteers and patients with acute coronary syndromes who were taking aspirin (asp) and clopidogrel (clop).

Figure 4. P-selectin expression on platelets in blood from healthy volunteers and patients with acute coronary syndromes measured using the P2Y12 P-selectin test or the aspirin P-selectin test. The data for the patients is separated for those who did and did not experience a further primary thrombotic event within 12 months of the tests being performed.
commercial tests: light transmission aggregometry (LTA), Multiplate, VerifyNow and Biocytex VASP. We found (Figure 5) that three of the aspirin tests used (P-selectin, LTA and VerifyNow) clearly identified three patients as being either non-responders to aspirin or non-compliant with therapy. Multiplate identified only one of these.

All of the P2Y12 tests (Figure 6) demonstrated variability in the effectiveness of clopidogrel to inhibit platelet function. Prasugrel treatment resulted in significantly greater inhibition than clopidogrel. Three patients with very high P-selectin values were subsequently found to be not taking prasugrel and high values were also obtained using the four other P2Y12 tests. The Spearman correlations (r values) obtained when the five different P2Y12 tests were compared are shown in table 1.

We looked to see whether the results obtained using the aspirin test or the P2Y12 test in ACS patients taking clopidogrel (n = 80) or prasugrel (n = 71) were related in any way to known vascular risk factors in the patients [6]. For data obtained using the P2Y12 test, as expected, P-selectin was higher in those taking clopidogrel than those taking prasugrel. However, this was only true in patients who did not have hypertension (606 [interquartile range (IQR) 392-794] versus 356 [IQR 294-455], p < 0.001). Values were significantly higher in those taking prasugrel (463 [IQR 323-557]) who had hypertension compared with those without hypertension (p = 0.017) and values almost reached those seen in the patients with hypertension taking clopidogrel (543 [IQR 397-793]). No associations with other risk factors were evident using the P2Y12 test. Also, there were no associations with any risk factors using the aspirin test. It was concluded that hypertension seems to negate the beneficial effects of prasugrel in some patients with ACS. Consequently platelet function testing might be useful to optimise therapy in this patient group.

3.2. Studies in patients undergoing surgery

A practical problem faced by surgeons is the potential for increased bleeding in cardiovascular patients receiving antiplatelet agents at the time of surgery, and in deciding whether to wait between stopping a drug and the surgical procedure. To gain some information on this clinical question we conducted a small observational study of platelet function in patients undergoing colorectal surgery [7].

Using our aspirin test we investigated the measured perioperative changes in platelet function in response to aspirin, and subsequently whether quantitative variations in platelet activity affected the severity and frequency of perioperative complications. 107 patients undergoing major colorectal surgery were recruited and assigned to either control (no antiplatelet therapy) or aspirin groups. P-selectin was measured following platelet stimulation at recruitment prior to cessation of medication, and at surgery before intervention. Perioperative complications, haemoglobin changes and blood transfusions were also recorded. Platelet function was higher in the control (n = 87) than in the aspirin group (n = 20) at recruitment (1303 [IQR 1102-1499] vs 77 [IQR 63.5-113.5], p < 0.01) and also at surgery (1224 [IQR 944-1496] vs 281.5 [IQR 106.8-943], p < 0.01). As demonstrated in figure 7 there was a positive correlation between the number of days of aspirin cessation and platelet function at surgery (r = 0.66, p < 0.01).

Complication rates and haemorrhagic complication rates (p < 0.05) were higher with aspirin than control, although complication severity was not increased. Platelet function of the entire cohort at surgery was not associated with complication rate, severity or transfusion use. Although complication rates were higher in the aspirin group, impaired platelet function within the ranges seen with aspirin continuation did not affect complication severity or rate or blood transfusion use. Consequently it was concluded that aspirin continuation may not affect clinical outcome in patients undergoing major colorectal surgery but that this needs to be confirmed in a larger study than the one we performed.

3.3. Studies in patients with acute stroke or transient ischaemic attack

Recently the range of antiplatelet agents used in patients after ischaemic stroke has been expanded with clopidogrel becoming a standard therapy to reduce the risk of further thrombotic events in these patients [2]. This raised the interest in assessing platelet function in stroke patients. At present, the aspirin kit and P2Y12 kit are being used in the
Figure 5. Comparison of various approaches to testing for effects of aspirin administered along with either clopidogrel or prasugrel. The approaches used were the P-selectin test with platelet stimulation using arachidonic acid and U46619, light transmission aggregometry (LTA), VerifyNow whole blood aggregation and Multiplate whole blood aggregation.

Figure 6. Comparison of various approaches to testing for effects of clopidogrel or prasugrel administered along with aspirin. The approaches used were the P-selectin test with platelet stimulation using ADP and U46619, light transmission aggregometry (LTA), VerifyNow whole blood aggregation, Multiplate whole blood aggregation and Biocytex VASP phosphorylation.
So far we have only been able to look at the baseline data obtained prior to randomisation [8]. Some of the patients admitted to the study following a stroke or TIA are already taking antiplatelet agents and we have been able to determine the effectiveness of existing antiplatelet therapies. The aspirin P-selectin test demonstrated that the group taking aspirin (without \( n = 125 \) or with dipyridamole \( n = 14 \)) had low platelet function (median 166 [IQR 131, 223] and 159 [IQR 137, 272] compared with 38 healthy controls (no drugs, median 1268 [IQR 983, 1542], \( p < 0.001 \), \( p = 0.049 \) respectively).

The P2Y12 P-selectin test showed that patients taking clopidogrel (\( n = 16 \)) had lower platelet function at 585 [352, 906] than those taking aspirin but not clopidogrel (\( n = 156 \)) at 1022 [772, 1233]

<table>
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<tr>
<th>P-selectin</th>
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**Table 1.** The Spearman correlations obtained when the five different P2Y12 tests (Figure 6) were compared.

![Figure 7. Results obtained using the P-selectin test with platelet stimulation using arachidonic acid following cessation of aspirin therapy in patients waiting to undergo major colorectal surgery.](image)

platelet function substudy of TARDIS (triple antiplatelets for reducing dependency after ischaemic stroke, NCT 01661322) to assess the effectiveness of several antiplatelet therapy regimes in patients following ischaemic stroke and transient ischaemic attack (TIA). TARDIS is an international multicentre trial that is testing the safety and efficacy of intensive antiplatelet agents (combined aspirin, dipyridamole and clopidogrel) versus guideline (aspirin and dipyridamole, or clopidogrel alone) given for one month following an acute stroke or a TIA. In the substudy, platelet function is measured at baseline and after 7 days of treatment with the prescribed therapy, and it is hoped to provide definitive information on the effectiveness of all the various treatments.
The main aim of the study was to evaluate the effects of an EP3 receptor antagonist on platelet function when used alone or in combination with a P2Y12 antagonist or a P2Y12 antagonist and aspirin. An EP3 antagonist may provide a new approach to the treatment of atherothrombotic disease by blocking the ability of prostaglandin E2 (PGE2) to promote platelet function. In addition to using the P-selectin approach to study platelet function we also measured platelet aggregation, again using a remote testing assay, and both approaches yielded very similar data.

First we quantitated the effects of DG-041 on platelet function in vitro and also its effects when administered in combination with the P2Y12 antagonist cangrelor, both without and with aspirin. Platelet aggregation and P-selectin expression were measured in whole blood (n = 10) following stimulation with the thromboxane A2 (TXA2) mimetic U46619 in combination with either the EP3 agonist sulprostone or PGE2. DG-041 alone partially inhibited platelet function in vitro, as did cangrelor. Addition of both DG-041 and cangrelor in combination provided significantly greater inhibition.

An ex vivo study was then performed using the same experimental approaches. Healthy volunteers (n = 42) were randomly assigned to receive no background treatment, clopidogrel or clopidogrel and aspirin for 10 days alongside DG-041 or placebo for 5 days, crossed over to placebo or DG-041 for the next 5 days. DG-041 partially inhibited platelet function ex vivo, as did cangrelor. Addition of both DG-041 and cangrelor in combination provided significantly greater inhibition. It was concluded that the antiplatelet effects of DG-041 and a P2Y12 antagonist used alone and in combination can be determined both in vitro and ex vivo.

PAMFix for assessment of platelet dysfunction

Mild platelet function disorders (PFDs) are complex and difficult to diagnose. The current gold standard test, light transmission aggregometry (LTA), including lumiaggregometry, is time and labour intensive and blood samples must be processed within a limited time after venepuncture. Furthermore, many subjects with suspected PFDs do not show a platelet abnormality on LTA. Consequently we assessed the diagnostic potential of an easy-to-use remote platelet function test as a diagnostic pre-test for dysfunction.

Women who are deemed to be at risk of complications of pregnancy are prescribed low dose aspirin in an attempt to avoid such complications. A small study was performed at the University of Liverpool to assess the effectiveness of aspirin in a series of treated patients using our aspirin kit alongside other tests. Initial results were very encouraging and a more extensive investigation is now in progress.

PAMFix for assessment of the effects of EP3 antagonists in combination with other antiplatelet agents

A study [9] was performed in which the measurement of P-selectin was one of the approaches used to investigate the effects of an EP3 antagonist, DG-041, administered to healthy volunteers as part of a drug development programme conducted in Iceland. The main aim of the study was to evaluate the effects of an EP3 receptor antagonist on platelet function when used alone or in combination with a P2Y12 antagonist or a P2Y12 antagonist and aspirin. An EP3 antagonist may provide a new approach to the treatment of atherothrombotic disease by blocking the ability of prostaglandin E2 (PGE2) to promote platelet function. In addition to using the P-selectin approach to study platelet function we also measured platelet aggregation, again using a remote testing assay, and both approaches yielded very similar data.

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suspected PFDs [10]. The remote platelet function test was compared with lumiaggregometry in participants recruited to the genotyping and phenotyping of platelets study (GAPP, ISRCTN 77951167). As for the other tests described above, whole blood was stimulated with platelet agonists, stabilized with PAMFix and returned to the central laboratory for analysis of P-selectin by flow cytometry. In this case, however, the levels of another platelet activation marker CD63 were also measured. Three different stimulants were used in these studies.

For the 61 study participants (42 index cases and 19 relatives) there was a good agreement between lumiaggregometry and the P-selectin test, with diagnosis being concordant in 84% of cases ($\kappa = 0.668$, $P < 0.0001$). According to both tests, 29 participants were identified to have a deficiency in platelet function and 22 participants appeared normal. There were four participants where lumiaggregometry revealed a defect but P-selectin did not, and six participants where P-selectin detected an abnormal platelet response that was not identified by lumiaggregometry.

In a further analysis of the data [11] nine subjects with impaired dense granule secretion were identified using lumiaggregometry (LA) and all nine patients presented with abnormal results on the flow cytometric assay. Two had a very high level of CD63 even in the unstimulated samples with reduced P-selectin, which is characteristic of Hermansky Pudlak syndrome type 2. Four subjects with the lowest levels of secretion on LA had reduced CD63 with all three stimulants and reduced P-selectin with

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**Figure 8.** Measurements of P-selectin and platelet aggregation in response to U46619 in combination with PGE$_2$. The volunteers treated with the EP3 antagonist DG-041 alone or together with clopidogrel or aspirin and clopidogrel. Values represent pre-drug administration (Pre), 12 and 4 hours after administration of DG-041 (DG12 and DG4) or placebo (P12 and P4).
aggregation proceeds. The fall in number of single platelets then provides a means of quantitating the extent of aggregation that has occurred. Platelet counting is performed using either a dedicated whole blood platelet counter such as the Ultra-Flo 100, or using a flow cytometer. Here PAMFix provides a convenient means of arresting the aggregation at the particular point or points of interest prior to platelet counting. The platelet count remains static for several hours after adding the PAMFix. At the University of Nottingham, fixation of blood has been very widely used as an aid to measuring platelet aggregation using the platelet counting approach [13-26]. When longer term stability is needed, for example when samples need to be transported before platelet counting, another fixative AGGFix can be used in place of PAMFix.

8. PAMFix and vWF binding
One use of PAMFix that has been recently reported to us is as a fixative for measurements of the amount of von Willebrand Factor bound to platelets in whole blood after stimulation with ristocetin (P. Gresele, personal communication).

9. PAMFix in canine lymphoma
Cancer in dogs is very common, with 1:4 animals being affected in their lifetime. Lymphoma is the at least one stimulant; this pattern was not observed in subjects with other types of platelet defects (n = 24). In another 3 subjects both CD63 and P-selectin were below the normal range with at least one agonist. This pattern of results was seen in 6 of 24 subjects who were labelled as mildly abnormal by LA. Only two subjects with normal platelet function as judged by LA (n = 28) had CD63 expression slightly below the normal range and P-selectin within the normal range. It was concluded that abnormal granule secretion can be identified using a new simple assay and it could be performed remotely as first-line diagnostic testing for all PFDs.

6. PAMFix for veterinary studies
Preliminary studies using blood from dogs to determine the effects of aspirin or a P2Y12 antagonist have been performed using the P-selectin approach (Figure 9), and findings similar to those made in humans have been obtained [12].

7. PAMFix in measurements of platelet aggregation
Platelet aggregation can be measured in whole blood and in PRP using a number of different approaches. One approach is to stir whole blood in the presence of a suitable platelet activating agent and count the number of single platelets in the blood as the aggregation proceeds. The fall in number of single platelets then provides a means of quantitating the extent of aggregation that has occurred. Platelet counting is performed using either a dedicated whole blood platelet counter such as the Ultra-Flo 100, or using a flow cytometer. Here PAMFix provides a convenient means of arresting the aggregation at the particular point or points of interest prior to platelet counting. The platelet count remains static for several hours after adding the PAMFix. At the University of Nottingham, fixation of blood has been very widely used as an aid to measuring platelet aggregation using the platelet counting approach [13-26]. When longer term stability is needed, for example when samples need to be transported before platelet counting, another fixative AGGFix can be used in place of PAMFix.

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Cancer in dogs is very common, with 1:4 animals being affected in their lifetime. Lymphoma is the
most common cancer to affect dogs representing about 14% of all canine malignancies. However, there are strong breed predispositions, the most noticeable being the Golden Retriever population of the United States. In this case, 60% of this breed will develop lymphoma during their lifetime.

Lymphoma in dogs is generally diagnosed by cytology or histology. However due to the availability of better prognostic data and speed, flow cytometry is increasingly being used throughout the US and Europe. One of the main difficulties encountered by laboratories using flow cytometry for this application is the stability of the sample during shipment. To obtain reliable results, the sample is needed at the laboratory within 24 hours of being taken from the dog. This is not always possible in veterinary medicine which is limiting the uptake of valuable flow cytometric testing in this sector.

PAMFix has therefore been used to investigate the possibility of stabilizing canine lymphocyte preparations in transit to the testing laboratory. Initial results are very encouraging. The investigators examined one lymph node aspirate and found the cell surface markers to be stable over a period of 4 days. They also found the cells to be stable over 24 hours on several other samples.

10. Conclusion
There is now considerable experience of using platelet activation markers, especially P-selectin, as a means of measuring platelet function. The approach is simple to use at the point that a blood sample is obtained and stabilization of the blood allows the sample to be sent to a central laboratory for analysis. This obviated the need for special equipment or technical expertise at the point of blood sampling.

CONFLICT OF INTEREST STATEMENT
PAMFix is produced and distributed by Platelet Solutions Ltd. which is a spin-out company of the University of Nottingham. All the authors of this paper have an association with Platelet Solutions Ltd. as follows: Stan Heptinstall (company founder, shareholder and director), Sue Fox (company founder and shareholder), Jane May (company founder and shareholder), Ann White (company employee) and Natalia Dovlatova (company employee and shareholder).

REFERENCES