Miniaturised Platelet Aggregation Assays using the NOVOstar Microplate Reader

BMG LABTECH

The Microplate Reader Company

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- A new platelet aggregation method is introduced using the NOVOstar microplate reader from BMG LABTECH
- Platelets aggregation in presence of agonists was supported by different shaking settings
- Data is in accordance with values obtaining using conventional low-throughput methods

Introduction

Platelets are small, anucleate blood cells derived from their precursor cells, megakaryocytes that are vital for haemostasis (1). Following blood vessel injury, subendothelial collagens are exposed to which platelets adhere. Platelet activation then ensues, culminating in platelet aggregation and the formation of a thrombus to stem the loss of blood. Under some pathological conditions such as the rupture of atherosclerotic plaques, however, the platelets are inappropriately activated resulting in the formation of platelet aggregates within the circulation. This frequently occurs at the site of atherosclerosis in coronary or carotid arteries triggering heart attacks and strokes respectively. The detailed characterisation of platelet function and underlying regulatory mechanisms is important for the development of new strategies to selectively reduce platelet reactivity and prevent thrombosis.

Platelet aggregation is a 'gold standard' assay of platelet function in the research and clinical laboratory setting (2). Platelet aggregation assays are performed using washed platelets or platelet rich plasma (PRP) in an optical aggregometer which measures light transmission through platelet suspensions during the aggregation process. Addition of an agonist such as collagen, results in aggregation and consequently an increase in transmitted light. Conventional platelet aggregometers require the use of large volumes of platelet suspensions (500 µL) and only limited number of reactions may be performed simultaneously. This method may therefore offer limitations when working with sparse or valuable samples, or species such as mice, where blood volume is limiting. In this application note we report the development of an alternative optical technique enabling the simultaneous analysis of a large number of samples in small volumes. This assay is performed using the NOVOstar microplate reader from BMG LABTECH.

Fig. 1: BMG LABTECH's multidetection microplate reader NOVOstar.

Materials and Methods

- White 96-well plates with transparent bottom, Costar, UK
- NOVOstar, BMG LABTECH, UK

Human platelet preparation

Human platelets were prepared as described previously (3). Briefly, blood was obtained from consenting drug-free donors immediately prior to use. 50 mL of blood were drawn into a syringe containing 3 mL of 4 % (w/v) sodium citrate and 7 mL of acid citrate dextrose (ACD: 2.5 % (w/v) sodium citrate, 2 % (w/v) glucose and 1.5 % (w/v) citric acid) were added prior to separation of the whole blood in 4 mL test tubes by centrifugation at 100 g for 20 minutes at room temperature to obtain PRP. Platelets were isolated from the PRP by centrifugation at 1400 g for 10 minutes in the presence of 50 ng/mL of prostacyclin (PGI2) to prevent platelet activation. The platelet pellet was resuspended and washed in 25 mL modified Tyrodes-HEPES buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 12 mM $NaHCO_3$, 1 mM $MgCl_2$, 20 mM HEPES and 5 mM glucose, pH 7.3) and 3 mL of ACD in the presence of 50 ng/mL PGI2 by centrifugation at 1400 g for 10 minutes. Finally, platelets were resuspended in 1 mL of modified Tyrodes-HEPES buffer and the platelet number was determined using a Z2 Coulter™ particle count and size analyser (Beckman Coulter) and the suspension volume adjusted to the required density ($4x10^8$ cells/mL). Platelets were rested at 30°C for 30 minutes prior to experimentation.

Platelet aggregation

The platelet suspension (100 µL/well) was pipetted into 96 well plates and then placed into the pre-warmed (37°C) NOVOstar. The injectors were connected to reservoirs containing modified Tyrodes-HEPES buffer and agonist [collagen related peptide (CRP)] separately. Control wells were injected with the equal volume of modified Tyrodes-HEPES buffer and all the samples were injected with an equal volume of CRP to a final concentration of 2.5 µg/mL. Conventional cuvette based assays include the stirring of samples to ensure platelet-platelet contact and therefore aggregation. In the present assay the shaking modes available on the NOVOstar were therefore used for a similar purpose. The linear, orbital and double orbital shaking modes were used and their suitability for aggregation studies compared. The following parameters were used to perform the aggregation assays in the plate reader. A conventional aggregation trace was also produced with 450 µL of platelets treated with 2.5 µg/mL CRP for 90 seconds using an optical aggregometer to compare the results.

Parameters

Measurement type:	Absorbance
Wavelength:	405 nm
No. of cycles:	30
Each cycle time:	19 seconds
Shaking width:	1 mm
Shaking mode:	Linear or orbital or double orbital
Additional shaking time:	9 seconds before each cycle
Temperature:	37°C

Results and Discussion

The aggregation assays were performed using 100 µL of platelets and 2.5 µg/mL final concentration of CRP in the 96 well micro titre assay plates using different modes of shaking. No aggregation was observed with linear shaking mode. However, a slow aggregation was seen with orbital shaking (figure 2).

Aggregation profiles that are similar to conventional aggregation traces were obtained using double orbital shaking (figure 3).

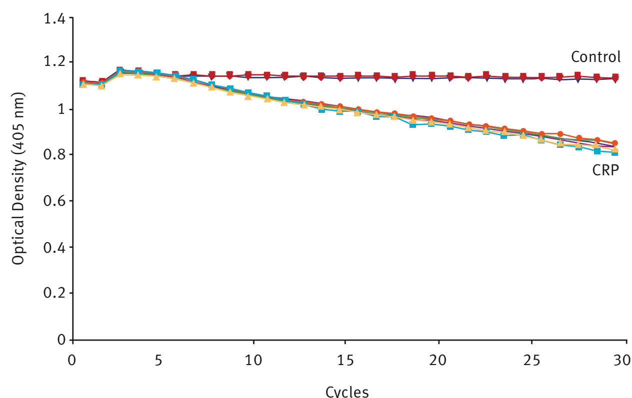

Fig. 2: Aggregation traces obtained in orbital shaking mode. 100 µL of platelets were treated with 2.5 µg/mL concentration of CRP or buffer control. Aggregation was monitored for 30 cycles (9 minutes 26 seconds in total) with orbital shaking for 9 seconds before each cycle with the parameters mentioned in the methods section. The traces with different colours show the repeats of the replicate samples from a single donor.

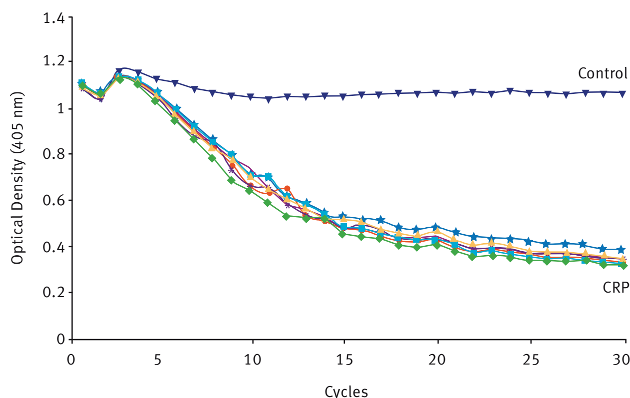

Fig. 3: Aggregation traces obtained in double orbital shaking mode. 100 µL of platelets were treated with 2.5 µg/mL concentration of CRP or buffer control. The aggregation was monitored for 30 cycles (9 minutes 26 seconds in total) with double orbital shaking for 9 seconds before each cycle with the parameters mentioned in the methods section. The traces with different colours show the repeats of the same experiment with reproducible results.

The aggregation in double orbital mode was reproducible when platelets from different donors were used (not shown). Other platelet agonists such as collagen and thrombin were also used to perform the aggregation assays using this methods and obtained results similar to conventional aggregation traces (figure 4).

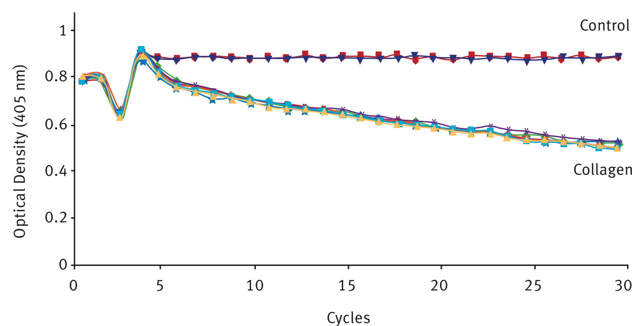

Fig. 4: Aggregation traces obtained in double orbital shaking mode using collagen. 100 µL of platelets were treated with 1 µg/mL concentration of collagen or buffer control. The aggregation was monitored for 30 cycles (9 minutes 26 seconds in total) with double orbital shaking for 9 seconds before each cycle with the parameters mentioned in the methods section. The traces with different colours show the repeats of the same experiment with reproducible results.

Conclusion

This assay offers capability to perform higher throughput aggregation assays using the NOVOstar plate reader. Due to the presence of two injectors, the dose concentration with agonist or antagonists may be performed simultaneously. The NOVOstar plate reader also incorporates a robotic pipette which can be used to transfer the reagents from a second plate or from the reagent container and thus this system allows the use of 3 different solutions simultaneously. This may enable the design of complex assays performed in an automated fashion.

References

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