

METHODS FOR UNDERSTANDING SHEAR INDUCED THROMBUS GROWTH

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ABSTRACT

The formation of blood clots is a key process in preventing major blood loss. Novel techniques are presented that allow for both the quantitative and qualitative analysis of shear dependent thrombus formation in real-time. Techniques that allow for scanned real-time images to be time corrected and reconstructed into a three-dimensional (3D) surface are demonstrated. As past work has demonstrated platelet activation and the subsequent thrombus growth is highly shear sensitive (Nesbitt, 2009). In order to attain better understanding to the underlying mechanisms driving thrombus formation the novel techniques presented allow for the shear history of individual platelets to be correlated with factors such as overall thrombus growth. A combination of the methods presented provides a powerful tool towards analysing thrombus growth in a highly temporal and spatially accurate manner.

INTRODUCTION

The formation of a thrombus (blood clot) in normal haemostasis and after vascular injury is an important process for prevention of blood loss. Platelets are an essential component of this process however the underlying biological and mechanical mechanisms causing platelets to aggregate and form a mature thrombus are not clearly understood. Such knowledge is fundamental in the development of future anti-platelet therapies. The cellular responses during thrombosis have for a long time been the primary focus of knowledge in this field, including the role of shear forces in activating a thrombotic response (Nesbitt, 2009; Tolouei, 2011). Platelets rapidly respond to changes in shear stress within a blood vessel (Nesbitt, 2009) and shear-modulated ligand engagement of platelet receptors trigger activation of intracellular signalling pathways that enable platelet adhesion, release of secondary platelet agonists and activation of platelet metalloproteinases to regulate receptor levels (Al-Tamimi, 2012). We now examine thrombus formation on a molecular level and correlate the

contribution of fluid shear to these molecular processes in unprecedented detail.

The current work analyses, in an experimental (*in vitro*) and numerical (*in silico*) environment, the fluidic dynamics around thrombus geometries. Distinct techniques are developed to examine both shear-dependent thrombus growth and retraction with high spatial and temporal resolution. These techniques permit the local quantification of thrombus aggregation characteristics across an entire thrombus field (300 μm scale) with resolution matching an individual platelet (2 μm scale). Such quantification enables relationships to be established between the biologically shear-sensitive receptors present on the platelet surface with the time resolved thrombus growth based on the Lagrangian shear history that individual platelets experience.

METHODOLOGY

Flow Loop

Human whole blood was pulled through a 0.2 mm x 2.0 mm collagen coated micro channel after being stained with a fluorescent dye DiOC₆ to enable visualisation of platelets. A Harvard Syringe Pump was used to drive the flow of blood through the micro channel for 3 minutes at a flow rate of 1.44 ml.min⁻¹ generating a wall shear rate (γ_w) of 1800 s⁻¹. Platelets subsequently adhere to the collagen layer and form thrombus aggregations over the 3 minutes of blood flow. The resulting thrombus aggregations were imaged in real time through a confocal microscope (Nikon A1) utilising 0.7 μm thick slices and a pixel density of 0.63 $\mu\text{m}/\text{pixel}$. An ultrasonic flow probe was used to measure the mean blood flow profile entering the micro channel

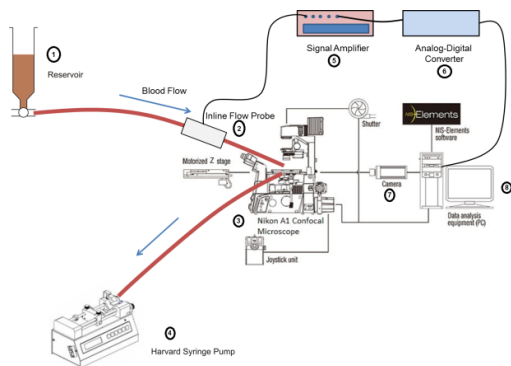


Figure 1. The flow loop utilised during the *in vitro* experiments. Blood (1) is pulled by the syringe pump (4) through a collagen coated micro channel. The mean blood flow profile was recorded using an ultrasonic inline flow probe (2). Real-time thrombus growth is captured through a confocal microscope (3) for the duration of the 3 minutes of blood flow.

Image Segmentation

The real time images acquired from the confocal microscope are vertically stacked and converted to 8-bit images for a greyscale threshold to be applied across all images in the stack.

Segmentation software developed in-house was then used to determine individual thrombus boundaries which were then isolated into separate slices which are collated vertically to reveal a point cloud in 3D space. A 3D surface is generated to encompass all the points contained within the point cloud using the software package Avizo (2015).

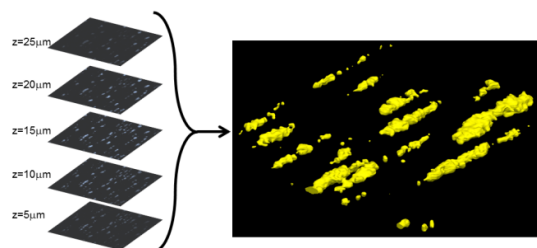


Figure 2. Three dimensional reconstruction of a thrombus field from the corresponding stack of 2D real-time acquired confocal slices

Time-Corrected Thrombus Field

The confocal microscope scans through a typical thrombus (approximately $75 \mu\text{m}$ (L) \times $12 \mu\text{m}$ (W) \times $42 \mu\text{m}$ (H)) using $0.7 \mu\text{m}$ slices in approximately 3.5 seconds. Over the period of time it takes to image a single thrombus from the top to bottom, the thrombus geometry is continuously growing. In order to accurately represent the changes to a growing thrombus surface, this period of time must be accounted for by producing a time-corrected image stack. To accomplish this, an algorithm was

developed which morphologically produces the intermediate confocal slices at any given slice in the stack, shown in Figure 3. A modified active contour level set method is used to generate the intermediate slices between any two given points in time (Chan, 2001). The thrombus boundary around the initial time point is defined as the initialising contour. The curves are transitioned over time to reveal the thrombus at all the intermediate time points. Each intermediate slice is allocated a real-time value in correspondence with the actual shutter timing from the confocal microscope. Additionally there is a time delay due to the time it takes for the microscopes piezo-z stage to move from the top slice to the bottom slice which needs to be accounted for. The algorithm takes this delay into account through the use of the position data for the stage over time.

The use of this technique reduces the number of usable acquired image stacks by two and consequently the overall growth period by 3.5 seconds. As the level set requires two individual images, time-corrected slices cannot be produced for times before the end of the first stack and for time points after the first slices of the last stack. This particular limitation is shown in Figure 3 in further detail, where time-corrected image stacks cannot be determined for times less than 3.5 seconds or after 4 seconds. Only two time stacks are shown for clarity on the process and limitations, however the number of image stacks is not a limitation for this technique.

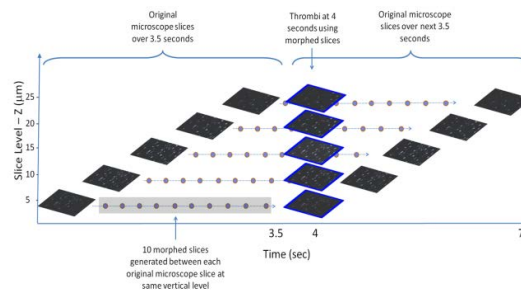


Figure 3. The process of time correcting the thrombus field using two time consecutive image stacks to resolve the thrombus field at the intermediate time points.

Growth Calculation

The capacity to determine both the real-time spatial position and volume of growth for a particular thrombus is of novel significance. Such knowledge would give insight into the underlying mechanisms which drive thrombus formation. The growth of a thrombus is calculated as the Euclidian distance between the thrombus surfaces at two subsequent points in time. In order to account for consolidation or embolism, the algorithm also determines distances to points on the surface which may be inside of the original surface. The algorithm begins by generating surface normal for each point on the triangulated surface mesh. The surface normals are projected up to the point in space where they intersect the thrombus surface at the next time point. In the case where no intersection is

detected, the direction of the normal is reversed as the intersection point must be within the original surface itself representing negative growth. The corresponding spatial distances are mapped onto the original surface shown in Figure 6, where red represents regions which have shown thrombus growth, while blue represents regions on the thrombus surface that have retracted or undergone embolism.

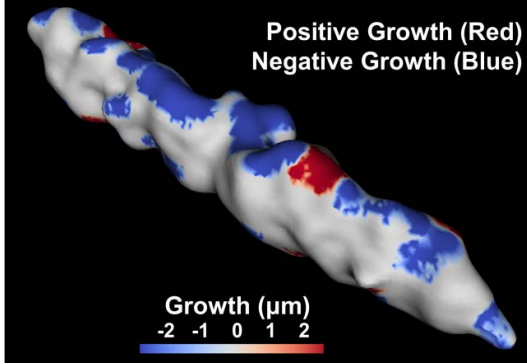


Figure 4. Growth and consolidation over a period of one second during the growth phase of the thrombus. Blue represents negative growth while red represents positive growth.

Numerical Technique

The experimental surfaces captured by the confocal microscope in real-time must have the blood flow simulated in order to determine the shear that the thrombus undergoes during the 3 minute period of blood flow. The numerical model shown in Figure 4 preserves the same Reynolds number (Re) as that used in the *in vitro* experiments where $Re=4.2$ (using channel height). The model also maintains the same aspect ratio (AR) to that found in the collagen coated microchannel, where,

$$AR = \frac{W}{H} \quad (1)$$

where W and H are the width and height of the channel respectively. Within the numerical model the length of the channel is reduced to 60 mm instead of the 100 mm found in the *in vitro* experiments in order to reduce the computational resources required. The OpenFOAM[®] package utilises the SIMPLE algorithm to attain a solution to the incompressible Navier-Stokes equations to steady state (OpenFOAM, 2014).

Within the numerical domain the reconstructed thrombus geometry is placed at the centre of the channel in order to avoid any wall effects. The overall configuration of the numerical domain is shown in Figure 4. The inlet to the channel has a Neumann pressure boundary with fully developed parabolic velocity profile with a mean velocity of 0.06 ms^{-1} , as recorded by the ultrasonic flow probe used in the *in vitro* experiments. The wall shear rate (γ_w), which was maintained at 1800 s^{-1} , is defined by,

$$\gamma_w = \frac{6Q}{WH^2} \quad (2)$$

where Q is the mean flow rate through the micro channel, W and H are the width and height of the micro channel respectively. The outlet has a zero pressure Dirchelet boundary condition with a Neumann boundary for velocity. The bottom, top and sides of the numerical domain in addition to the surface of the thrombus have a zero velocity Dirichlet boundary condition. The local shear rate throughout the numerical domain is determined using,

$$\gamma_{local} = \sqrt{2 \times D:D} \quad (3)$$

where D is the strain rate tensor with U the velocity vector field,

$$D = \nabla U + (\nabla U)^T \quad (4)$$

The mesh within the numerical domain consists of a polyhedral finite volume mesh with a refinement region located $200 \mu\text{m}$ radially around the centre of the channel to encompass the thrombus geometry. A typical mesh of the domain utilises approximately 9 million elements, with 8.4 million elements required to mesh the complex surface geometry of the thrombus. A grid resolution study was completed until the total difference in velocity and pressure fields between grids was below 0.1%. A steady state solution was attained to the numerical domain with the final pressure and velocity residuals reducing to below 10^{-10} .

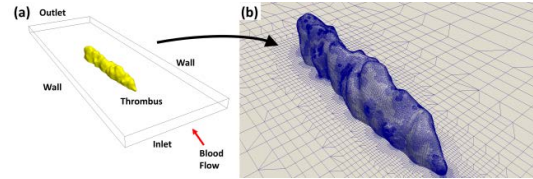


Figure 5. a) Model used in the numerical technique with the thrombus located at the center of the channel. b) Overall mesh used to resolve the thrombus surface and surrounding refinement regions.

SHEAR CORRELATION

The combined approach of using the shear a thrombus aggregation experiences correlated with the corresponding growth provides a very useful tool in analysing the mechanisms which influence thrombus growth under different hemodynamic conditions. The shear forces individual platelets experiences through interaction with the thrombus aggregation surface is determined through the numerical solution to the *in silico* flow field. Consequently a thrombus interaction region (TIR) is defined as the region around a growing thrombus where the changes in local shear gradients are due to the individual platelet interacting with the thrombus surface. The TIR is taken as a distance which is 10% of the thrombus length in the direction of the flow. In order to evaluate the shear history of each individual platelet adhering to the thrombus surface, every point on the surface (a representative thrombus contains approximately

27, 000) is used to calculate a path line in the opposite direction to the flow. Each path line is generated through the integration of the velocity field through the use of a fourth-order adaptive Runge-Kutta method to reveal the spatial path taken by each individual platelet in addition to the shear experienced at each point along the platelets travel path (Press, 2007). Error along any one path line is maintained below 10^{-8} . As the thrombus surface itself has a Dirichlet boundary condition the velocity at the thrombus surface by definition would be zero which raises the issue of the path lines being unable to commence from a location where the velocity is zero. Accordingly every surface point is displaced $1 \mu\text{m}$ along each surface normal, allowing for path lines to be calculated. Using points that are $1 \mu\text{m}$ above the thrombus surface would represent a platelet which is deemed to have already made contact with the thrombus surface as the radius of a platelet is approximately $1 \mu\text{m}$. Various properties along each path

By using CFD to calculate the flow field in comparison to other techniques such as particle image velocimetry (PIV) it provides the advantage of being able to determine the Lagrangian properties of the flow and consequently determine the shear experiences by platelets interacting with the thrombus and other platelets. Figure 6a) depicts the path taken by an incoming platelet towards a growing thrombus geometry into a region of high shear. The thrombus surface is coloured with the shear $1\mu\text{m}$ normal to the thrombus geometry. The Lagrangian shear history of a platelet during the last 0.15 seconds prior to adhesion is shown in Figure 6b). The shear history reveals the platelet begins to experience significant shear gradients only within the last 0.1 seconds prior to adhesion and whilst within the TIR. In conjunction with previous studies, the peak shear experienced in the immediate region around a growing thrombus is vastly higher than the wall shear rate (Butler, 2012). During the

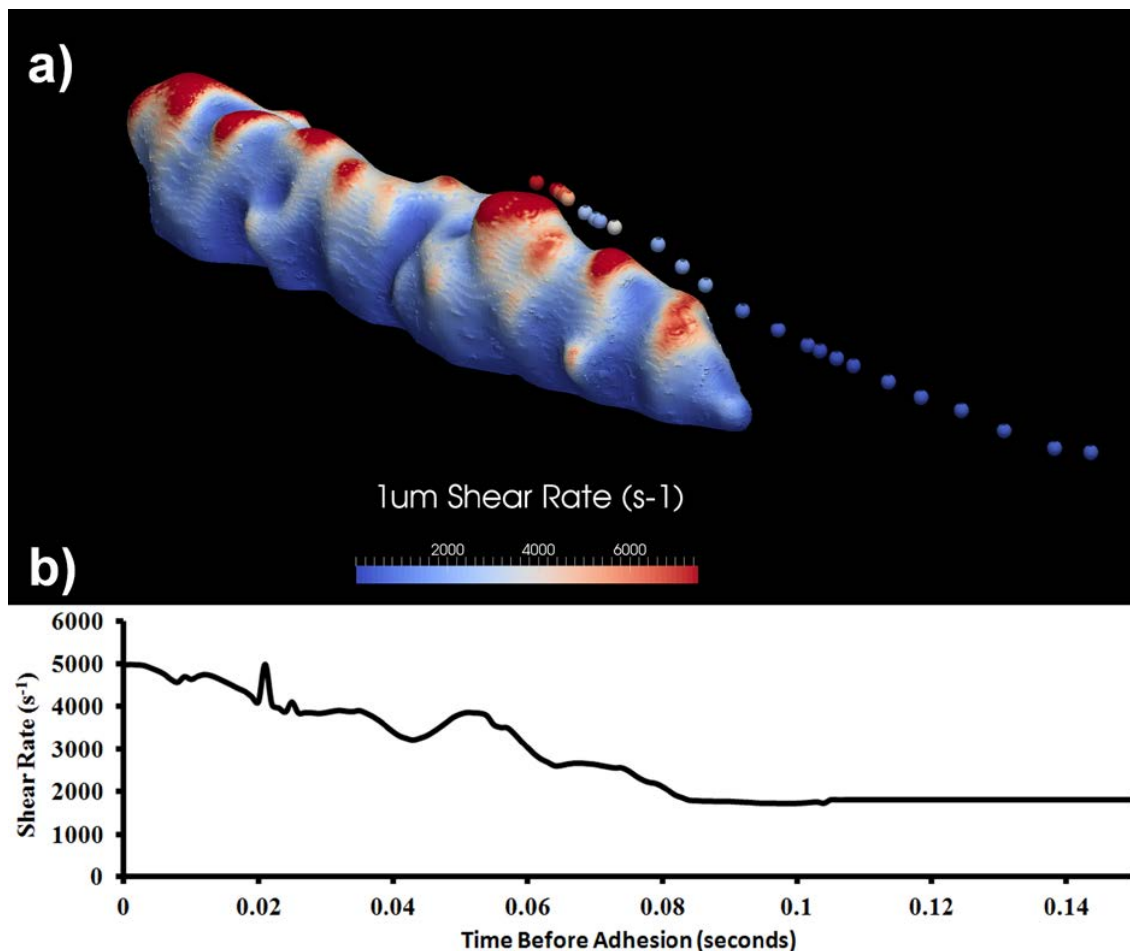


Figure 6. a) Shear rate (s^{-1}) $1\mu\text{m}$ above the thrombus surface mapped onto the original surface. The individual spheres represent the trajectory taken by platelets which are also coloured based on shear rate experienced. b) Shear rate along the platelet path shown with respect to time. The platelet shown enters the TIR 0.1 seconds prior to adhesion.

line, which are representative of the hemodynamic forces platelets experience moments preceding adhesion, can then be mapped onto the surface of the thrombus.

last 0.1 seconds there is a significant increase in shear rate experienced by the platelet (an increase of over 170%). Subjecting platelets to such great shear gradients are believed to commence platelet activation (Nesbitt, 2009).

CONCLUSION

A novel approach aimed at investigating platelet adhesion and thrombus growth is presented through both *in vitro* and *in silico* experiments. The ability to correlate the shear experienced by individual platelets prior to adhesion and the subsequent thrombus growth is of great significance. The capacity to determine parameters such as thrombus growth and platelet shear in a spatially and temporally accurate manner provides a unique capability towards attaining a greater understanding the highly shear dependent nature of thrombus formation.

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