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PLATELET ADHESION TO POLYURETHANE UREA UNDER PULSATILE FLOW CONDITIONS

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Abstract

Platelet adhesion to a polyurethane urea surface is a precursor to thrombus formation within blood-contacting cardiovascular devices, and platelets have been found to adhere strongly to polyurethane surfaces below a shear rate of approximately 500 s^{-1} . The aim of the current work is to determine platelet adhesion properties to the polyurethane urea surface as a function of time varying shear exposure. A rotating disk system is used to study the influence of steady and pulsatile flow conditions (e.g. cardiac inflow and sawtooth waveforms) for platelet adhesion to the biomaterial surface. All experiments retain the same root mean square angular rotation velocity (29.63 rad/s) and waveform period. The disk is rotated in platelet rich bovine plasma for two hours with adhesion quantified by confocal microscopy measurements of immunofluorescently labeled bovine platelets. Platelet adhesion under pulsating flow is found to exponentially decay with increasing shear rate. Adhesion levels are found to depend upon peak platelet flux and shear rate regardless of rotational waveform. In combination with flow measurements, these results may be useful for predicting regions susceptible to thrombus formation within ventricular assist devices.

Keywords

platelet adhesion; fluid dynamics; polyurethane; pulsatile flow; rotating disk; shear stress

Introduction

The success of biomedical devices that contact blood may be limited by thrombosis and the subsequent embolic complications that arise from this process. Virchow's Triad of flow, material and blood constituency provide the fundamental understanding of thrombosis. Blood-material interaction follows a complex interwoven pathway involving plasma protein adsorption and platelet and leukocyte activation. Platelet activation is initiated by binding to proteins adsorbed on a material surface. Activation leads to physiologic responses such as granule content release, P-selectin expression, shape change promoting platelet-platelet contact, synthesis of prostaglandins and thromboxane B_2 , and formation of platelet

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microparticles. While the intricacies of these responses are not completely understood, adherent platelets are known to be coagulant in nature¹.

Although thrombosis is a particularly difficult problem for ventricular assist devices (VADs), and is the basis of the current work, platelet adhesion represents a challenge for devices including vascular grafts, stents, heart valves, intravascular catheters, oxygenating membranes, and hemodialysis components. Resulting complications may include obstruction, thrombotic occlusion, or embolization and may lead to fatality or costly intervention¹. Substrate alterations such as treatment with polyethylene oxide and/or surface modifications have proven successful in lowering protein adsorption and platelet adhesion in some instances^{2,3,4}; however, extended blood exposure remains a risk factor for growth. In order to sustain physiologic outputs while limiting risk of device-related thrombosis, platelet adhesion characteristics should be well understood.

Polyurethanes are often used for blood contacting components because of their high levels of hemocompatibility and fatigue resistance⁵ with polyurethane urea (PUU) used as the blood sacs with the 50 cc Penn State LVAD. Thirty day animal trials with the Penn State 50 cc VADs showed thrombus formation in several regions of the pump, a phenomenon not seen in larger devices (> 70 cc).^{6,7,8} Since the PUU surface material and topography is essentially the same in the 50 and 100 cc devices, the change in the local fluid mechanics that the biomaterial surfaces are exposed to must play a major role in thrombus formation within a 50 cc LVAD.⁹ Hochareon *et al.*, using particle image velocimetry (PIV), found strong correlations between *in vivo* clot deposition and regions where wall shear rates remained low throughout the cardiac cycle of the 50 cc LVAD.⁶ Previous studies using a parallel-plate flow chamber and epi-fluorescent video microscopy confirmed that increasing wall shear rates reduced the ability of platelets to adhere to a polyurethane surface. Stable platelet adhesion was found to be markedly reduced when increasing a steadily applied shear rate from 100 s⁻¹ to 500 s⁻¹.¹⁰

The 50 cc device has been studied in an *in vitro* setting over the previous two decades using PIV. Previous experiments matched LVAD operating conditions to those found clinically by means of a mock circulatory system.¹¹ Wall shear rates have been found to remain below 500 s⁻¹ for appreciable amounts of time for multiple design iterations in several regions of the pump.^{12,13,14} Alterations in the mitral valve orientation were found to improve the duration of wall washing in thrombus-prone regions; however, the bottom of the pump near the outlet port remained susceptible.¹⁵

A thrombus susceptibility potential (TSP) was developed through a computational study of the 50 cc Penn State LVAD to objectively compare parametric study results.¹⁶ The metric relates calculated wall shear rates to several shear variables including threshold for thrombus deposition, inhibition of platelet deposition, and exposure times. The TSP has been used by Roszelle to quantify the probability of thrombosis in weaning and valve orientation studies of the Penn State pediatric VAD¹⁷ and more recently by Navitsky *et al.* to compare two 50 cc LVAD design geometries.¹³ These studies used a value for thrombus deposition threshold (500 s⁻¹) from Hubbell and McIntire¹⁰ and a shear value for inhibition of platelet deposition (1000 s⁻¹) from Balasubramanian,¹⁸ although they are not specific to platelet interaction

with PUU. The current work helps define these values for use with PUU with particular interest how a pulsating flow influences platelet adhesion.

We use the rotating disk system (RDS)²² shown in Figure 1A as a means to study adhesion characteristics through a well-defined flow field near the surface of the disk.^{19,20} The shear rate along a radial line extending outward from the center is easily defined as velocity increases linearly with radial distance. A RDS similar to the one used here has been previously used for biomedical applications. Wang *et al.* used the system to measure platelet mediated adhesion of *staphylococcus epidermis* to a hydrophobic polymer surface over a shear range of 0 – 4867 s⁻¹.²¹ More recently, Milner *et al.* studied properties of platelet adhesion to varied texture PUU surfaces over a range of 0–4467 s⁻¹.²²

The purpose of this study is to understand the effect that pulsatility has on platelet adhesion on a smooth PUU surface and determine if platelet flux and the adhesion coefficient must be calculated differently under pulsatile flow using the RDS system. Previous platelet adhesion studies used only steady flow to understand the mechanisms and characteristics but with the previously referenced 50 cc device, or any device placed in the cardiovascular system, the flow is pulsatile. Finally, we address if our previous TSP coefficients based on others' work is applicable to the smooth PUU used in the 50 cc device. To this end, we examine the effects of peak shear exposure for time varying waveforms on platelet adhesion to a smooth PUU surface. Experiments are conducted over the physiologically relevant shear rate range of 0–933 s⁻¹. Particular attention is given to the adhesion levels below 500 s⁻¹, as the effects of peak exposure and pulsatility at low RMS shear values are not well understood. The results provide better understanding of platelet adhesion under low flow and pulsatile conditions and help improve the TSP for use with the smooth PUU.

Materials and Methods

Smooth PUU materials

PUU samples were prepared by successively spin casting and curing 18% Biospan MS/0.4 on a smooth PDMS mold three times. The first layer was spun at 1500 rpm for 60 s to create a very thin, smooth layer. The second layer was created by spin casting at 800 rpm for 60 s and the third layer was spun at 400 rpm for 60 s. The sample was cured overnight in a vacuum at room temperature following each successive casting. This process was followed to slowly increase the thickness of the material while maintaining smoothness and reducing bubble formation. The PUU material was cut to a 20 mm diameter and mounted on a 20 mm metallic disk scribed with concentric circles spaced 1 mm apart and 6 radial lines separated by 60° (Figure 1A).

Platelet Rich Bovine Plasma Preparation

Whole bovine blood was drawn from the jugular vein of healthy specimens at the Penn State dairy barns according to IACUC #31641. Whole blood was anti-coagulated with CPDA-1 and subsequently centrifuged at 600 g for 12 minutes with 1% acceleration and deceleration. CPDA-1 has been shown to leave platelet function and viability unaltered especially when stored for a short time period at room temperature.^{23,24} Platelet rich plasma (PRP) was

gently separated from the red cell population and buffy coat and transferred into a clean 50 ml centrifuge tube. Platelet concentration measurements were made using a hemacytometer (Reicher, Buffalo, NY) and bright-field microscope (Nikon, Melville, NY) with associated 40 $\times$ lens (Nikon, Melville, NY). Bulk platelet concentration (C_{∞}) was adjusted to 350×10^6 platelets/ml. The plasma was measured to have an average kinematic viscosity of 1.55 cSt at 30 $^{\circ}$ Celsius using a viscoelastic analyzer (Vilastic, Austin, TX). Plasma was then transferred to a 100 ml PTFE beaker and maintained at 30 $^{\circ}$ Celsius throughout the experiment by a hot plate.

Rotating Disk System

The RDS (Pine Instruments, Grove City, PA) (Figure 1A) was used to deliver a steady flow and four pulsatile flows (cardiac inflow and three different ramp waveforms) to the PUU surface. Waveforms were produced from a programmed voltage delivered by a function generator (Agilent, Santa Clara, CA). Just prior to rotation, the PUU coated disk was lowered approximately 3 mm into the PRP. All waveforms (Figure 1B) had the same root mean square (RMS) angular velocity of 29.63 rad/s and each experiment was performed 6 times. First, a steadily applied voltage was used to produce a baseline steady rotation of 29.63 rad/s. A cardiac inflow waveform obtained from the Penn State 50 cc LVAD operating at 86 bpm was then used to study the effects of pulsatility on platelet adhesion. The inflow waveform reached a peak angular velocity of 54.35 rad/s with an acceleration of 488 rad/s². Then, the effects of peak shear were tested by applying three ramp waveforms, which had different peak angular velocities while retaining the same RMS velocity of 29.63 rad/s. The waveforms were programmed to have a 90% rise and 10% fall of the total cycle. The first peaked at the same peak angular velocity as the cardiac pulse waveform (54.35 rad/s, acceleration = 91.4 rad/s²) as a baseline for comparison. The second waveform reached a peak 25% lower (40.76 rad/s, acceleration = 38.1 rad/s²) than the baseline ramp waveform. The third reached a peak 25% higher than the baseline ramp waveform (67.93 rad/s, acceleration = 149.4 rad/s²). All pulsatile waveforms maintained the same period of 700 ms.

Experimental Validation of Quasi-Steady Flow and Shear Rates

We can use the well-developed theory for steady rotation to calculate the wall shear rates, provided that each of the flow fields can be shown to be quasi-steady. Laser Doppler velocimetry (LDV) was used to test this assumption – that the fluid velocity adjusts instantaneously to the acceleration of the disk. An acrylic cup was manufactured with the same dimensions as the PTFE cup in Figure 1A, and an analog fluid (37% H₂O, 63% NaI, by weight) was created that matched the kinematic viscosity of the PRP and the refractive index of the acrylic. One dimensional velocity data using LDV (TSI, Inc., Shoreview, MN) was collected at radial locations between 3 and 7 mm, approximately 100 μ m beneath the disk surface, for each of the waveforms we used, plus an additional one that produced an angular acceleration of the same magnitude as the high, negative acceleration in the last 70 ms of the +25% ramp (Figure 1B). The only velocity considered was the theta component in a cylindrical coordinate system, which was assumed to dominate the flow near the disk surface. The period of the waveforms was separated into bins of approximately 10 ms each for analysis of the velocity over the entire cycle. Mean and standard deviations were

calculated for each bin separately, and velocity measurements were filtered using two standard deviations.

As mentioned previously, the peak positive accelerations for the cardiac waveform, baseline ramp, -25% ramp, and +25% ramp were 488, 91.4, 38.1, and 149.4 rad/s², respectively. In addition to these waveforms, an extra ramp waveform was used in the LDV experiment that produced a disk acceleration of 1345 rad/s², which is equal in magnitude to the high, negative acceleration briefly applied to the disk at the end of the +25% ramp. The LDV results confirmed that the quasi-steady assumption was valid over the entire range of disk accelerations encountered in the platelet adhesion study, and thus, all shear rate calculations can be made using the steady rotation rate theory at the appropriate temporal rotation rate.

Not only was LDV used to verify the quasi-steady assumption was valid, but it was also used to validate the theoretical shear rate calculations. Using a steady rotation rate of 283 RPM, which is equivalent to the RMS angular velocity of the ramp functions, the theta component of velocity was collected 100 μ m beneath the disk surface at radial locations 0 to 10 mm from the center of the disk. Based on the steady state solution provided by Benton¹⁹, we determined that the decrease in velocity followed a linear curve with an R² correlation coefficient greater than 0.99 from 0 to 100 μ m beneath the disk surface. Consequently, the experimental shear rates could be calculated by equating the wall normal velocity gradient to the change in velocity from a depth of 0 to 100 μ m beneath the disk surface divided by 100 μ m. The experimental shear rates agreed with the theoretical values for radial locations up to 9 mm, at which point edge effects disturbed the flow.

Quantification of Platelet Adhesion

Following the rotation, platelets are removed from the PUU suspension by 6 aspirations of 35 ml phosphate buffered saline (PBS). PBS is then replaced with a 1% para-formaldehyde (PFA) solution for one hour to fix the platelets. PFA is then replaced by PBS and allowed to equilibrate for 10 minutes. The disk, with adhered PUU, is then detached from the RDS. For each disk, a confocal microscopy image is acquired at the disk center and at the intersection of each concentric circle along three radial lines for a total of 28 images per disk (Figure 1A). The platelet count at the disk center, along with the average number of platelets for three randomly chosen radial intersections, constitutes one experiment. Imaging is conducted using a FV- 1000 confocal microscope (Olympus Microscopes, Shinjuku, Tokyo) and associated 100x dry objective (Olympus Microscopes, Shinjuku, Tokyo) of immunofluorescently labeled bovine platelets following treatment with a primary CAPP2A mouse anti-bovine α IIb β 3 antibody (VMRD, Pullman, WA) (1.5 μ l CAPP2A+1 ml 6% donkey serum (Sigma-Aldrich, St. Louis, MO) and secondary Alexa-Fluor 488 donkey anti-mouse IgG (Invitrogen, Eugene, OR) (1.25 μ l +1 ml 6% donkey serum). In a preliminary experiment, primary antibody was withheld to confirm secondary antibody specificity; no fluorescence was observed. There were variations in ACs at the disk center but these were in line with results found by Milner *et al.*²² The interrogation region size examined under confocal microscopy using a 100x objective was 0.011187 mm² leading to a shear variation of 5.48 s⁻¹ from image center to image edge.

The adhesion coefficient (AC), percentage of available platelets binding to the surface, is defined as:

$$\frac{N}{j \cdot t} * 100\% \quad [1]$$

where N is the average number of platelets per unit area, j is the mass flux (or rate of platelet flow per unit area), and t is the rotation time.¹⁸ The flux is defined as:

$$j = 0.62 D^{2/3} \omega^{1/2} C_{\infty} \nu^{-1/6} \quad [2]$$

where ω is the RMS angular velocity, C_{∞} is the bulk concentration, ν is the kinematic viscosity. The diffusivity (or diffusion coefficient, indicative of diffusion mobility) (D) is defined as:

$$D = \frac{K_B T}{6\pi\eta b} \quad [3]$$

where K_B is the Boltzmann constant, T is the absolute temperature, η is the dynamic viscosity of PRP, and b is the average platelet radius. (Platelets vary in size, both in activated and un-activated form, a 2 micron radius was chosen as a representative value to use as a constant in the calculation of D). Solid body rotational velocity (v) at each point along the disk surface are solved for theoretically by linearly equating:

$$v = \omega r \quad [4]$$

where ω is angular velocity and r is radial distance from the disk center.

Results

Platelet adhesion is quantified for the steady, cardiac inflow, and ramp waveforms shown in Figure 1B. The RMS angular velocity, shared by all waveforms, is used for both the calculation of shear rate and the mass flux term within the AC calculation. Measurements for N are included in Table 1 and measurements for j and ω for each waveform are displayed in Table 2. For all experiments, adhesion levels were highest at the disk center corresponding to a shear rate of 0 s^{-1} . Here, ACs (as the percentage of possible adherent platelets) were 0.70 (steady), 0.50 (cardiac inflow), 0.47 (0% baseline ramp), 0.79 (−25% ramp), and 0.20 (+25% ramp). All waveforms decayed exponentially as a function of increasing shear rate with an R^2 correlation coefficient of 0.89 (steady), 0.73 (cardiac inflow), 0.90 (0% baseline ramp), 0.79 (−25% ramp), and 0.87 (+25% ramp). Even though we altered the waveform, the RMS for each was constant indicating that pulsatility does have an influence on the platelet activity.

Platelet adhesion was greatly reduced on polyurethane surfaces at shear rates above 500 s^{-1} .¹⁰ At the radial location corresponding to 518.15 s^{-1} , ACs were found to be 0.17 (steady), 0.08 (cardiac inflow), 0.05 (0% baseline ramp), 0.19 (−25% ramp), and 0.05 (+25% ramp). At the outer most radial location measured, corresponding to 933 s^{-1} , mean ACs had decayed to below 0.06 for all waveforms.

While there are large differences in ACs at the disk center, there was no statistical significance (one way ANOVA, 95% confidence interval (CI)) due to the large standard error at this point. The next four points measured, corresponding to shear rates of 103.6, 207.3, 310.9, and 414.5 s^{-1} , were found to be statistically different (one way ANOVA, 95% CI). This result indicated that platelet adhesion below 414.5 s^{-1} is directly affected by the peak shear exposure. The next location measured, corresponding to a shear rate of 518.15 s^{-1} showed no significant difference between waveforms. No significant difference was found at any of the remaining (higher shear rate) points examined. The correlation of AC and peak shear exposure is shown in Figure 2 for all waveforms following two hour rotation. Aside from one singularity (cardiac inflow waveform at 1544 s^{-1}), all AC calculations reach a low, asymptotic value below 0.1 beyond approximately 1000 s^{-1} . This data indicates that adhesion rarely occurs above 1000 s^{-1} .

Discussion

Platelet adhesion to a smooth PUU surface showed exponential decay as a function of increasing shear rate. For each experimental waveform considered, N was the highest at the disk center, corresponding to an exposure of 0 s^{-1} . When plotted as a function of RMS shear rate and flux (j), ACs scaled according to the rotational waveform peak angular velocity below 414.5 s^{-1} . When plotted as a function of peak shear rate and flux, AC waveforms effectively collapsed upon one another reaching an asymptotic level below 10% possible adherent platelets beyond $\sim 1000 \text{ s}^{-1}$.

The RDS provides a pulsatile model to study how LVAD inflow waveforms and resultant surface washing may affect platelet adhesion. The adhesion pathway is complex and has been studied extensively from a biochemical perspective. These experiments study the precursor, flow-mediated phenomenon prior to the initiation of this pathway. The subsequent events of adhesion on the PUU surface require future experimentation using whole blood. This work paves the way for such experiments by providing insight into the localization of primary events within a device. With this rationale in mind, peak platelet flux and shear rate were found to be influential in downstream adhesion.

The submicron structure of the PUU surface has also been shown to be influential upon adhesion levels²². Examination of the material surface was beyond the scope of this work; however, state of the art material preparation processes were followed to ensure a uniformly smooth surface. The PUU material tested within this study is used for many blood contacting biomedical applications, and as such, the results may be applied to these other devices. However, many of the LVADs used clinically today are axial flow pumps that deliver continuous, or slightly pulsating, flow through the device with different material surfaces. While thrombus formation is not considered a major problem for these axial flow devices, this work here may be helpful to understand the precursor phenomenon for thrombi found on those devices. Similar work was completed on a centrifugal pump focusing on the roughness of the surface and demonstrated the influence of platelet adhesion²⁵

Clinically, the alterations in the peak of the ramp waveforms used here can be considered as a model for altered operating conditions for a LVAD, especially for pediatric patients on

pulsatile devices²⁶. For example, an increase in flow from the onset of diastole followed by reduction during mid to late diastole may be used, similar to the waveforms used here to study platelet adhesion. By understanding how platelet adhesion occurs in pulsating flow, greater care can be taken to using LVADs clinically.

In this study, we used bovine blood/platelets, the same model with which many ventricular assist devices are first tested. Unfortunately, bovine platelets lack the open canalicular system (OCS) exhibited in human platelets and may exhibit altered secretory properties upon activation. This could lead to a different progression of thrombotic events, but since the focus here was the adhesive characteristics of platelets, this should not affect the results.²² In addition, these platelet adherence correlations on explanted blood sacs (fabricated from PUU) can be compared directly to PIV shear rate measurements made within the Penn State LVAD in previous studies, which will enable better thrombus prediction and design comparison.^{6,12,13,14}

Conclusion

Platelet adhesion to the smooth PUU surface used in many blood contacting applications, including the Penn State LVAD, was studied under pulsatile shearing conditions using a RDS system. Adhesion, under pulsatile flow, was found to be directly related to peak shear experienced in regions where RMS shear rates remained below 500 s^{-1} and to be greatly reduced where peak shear surpassed 1000 s^{-1} . This validated the shear values used to represent platelet inhibition within the TSP in previous work that was originally based on steady flow conditions.^{13,17} Here, we also found that platelets adhered to the greatest extent in regions of lowest shear on a polyurethane urea surface. The potential implications of adhesion characteristics below 500 s^{-1} need to be further correlated to deposition within *in vivo* model explants, but the results presented here strengthen the idea that ventricular assist devices should be optimized to reduce prolonged exposure to shear levels below 500 s^{-1} . The adhesion coefficient, when calculated as a function of peak angular fluid velocity, allows for a predictive capability of likely adhesion localization. This capability may prove especially useful within low shear models where only brief periods of washing occur.

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Figure 1.

A) Schematic representation of the RDS setup and the scribed metal mounting surface for the smooth PUU material. The disk is rotated by an applied voltage waveform from a function generator. Confocal images (0.011187 mm^2) are taken at each radial intersection. Image modified from Milner *et al.*¹⁵ B) Angular velocity (rad/s) for experimental waveforms over the course of one 700 ms period.

Figure 2.

Adhesion coefficient plotted versus shear rate calculated from peak angular velocity. The flux is also calculated based upon peak angular velocity.

Table 1
Average (and SEM) number of platelets measured at each radial location along the disk for each of the five experimental conditions.

Radial location (mm)	N (platelets/mm ²)				
	Steady	Cardiac Pulse	Baseline ramp	-25% ramp	+25% ramp
0	1638.8±509.2	1162.1±308.8	1102.5±385.3	1841.4±566.9	476.7±75.4
1	1306.1±419.2	943.6±419.5	730.0±242.3	948.5±136.0	327.8±41.4
2	1124.8±407.6	332.7±49.5	471.8±140.1	913.8±104.5	367.5±71.6
3	481.7±135.3	735.0±379.8	340.2±97.3	953.5±230.3	218.5±22.6
4	635.7±223.6	201.1±25.2	223.5±69.8	700.2±225.3	94.4±55.7
5	394.8±166.2	186.2±45.3	109.3±64.7	446.9±171.7	109.3±53.1
6	248.3±45.3	491.6±421.6	178.8±86.6	124.2±53.0	94.4±51.8
7	275.6±114.2	149.0±54.9	119.2±28.7	109.3±55.8	99.3±56.4
8	64.6±12.0	96.8±31.4	44.7±12.2	198.6±97.2	74.5±24.0
9	29.8±18.8	126.6±45.3	84.4±9.9	9.9±9.9	69.5±36.6

Table 2

RMS and peak values for both flux measurements and angular velocities for the five experimental conditions.

	Steady	Cardiac Pulse	Baseline ramp	-25% ramp	+25% ramp
j_{RMS} (platelets/s*mm ²)	32.1	32.1	32.1	32.1	32.1
j_{Peak} (platelets/s*mm ²)	32.1	43.5	43.5	37.6	48.6
ω_{RMS} (rad/s)	29.63	29.63	29.63	29.63	29.63
ω_{Peak} (rad/s)	29.63	54.35	54.35	40.76	67.93