Published in final edited form as:

J Biomed Mater Res B Appl Biomater. 2013 November ; 101(8): 1367–1376. doi:10.1002/jbm.b.32954.

Coagulation-induced resistance to fluid flow in small-diameter vascular grafts and graft mimics measured by purging pressure

Michael D. Nichols, Rewa Choudhary, Santhisri Kodali, and William M. Reichert

Department of Biomedical Engineering, Center for Biomolecular and Tissue Engineering, Duke University, Durham, North Carolina 27708

Abstract

In this study, the coagulation-induced resistance to flow in small-diameter nonpermeable Tygon tubes and permeable expanded polytetrafluoroethylene (ePTFE) vascular grafts was characterized by measuring the upstream pressure needed to purge the coagulum from the tube lumen. This purging pressure was monitored using a closed system that compressed the contents of the tubes at a constant rate. The pressure system was validated using a glycerin series with well-defined viscosities and precisely controlled reductions in cross-sectional area available for flow. This system was then used to systematically probe the upstream pressure buildup as fibrin glue, platelet-rich plasma (PRP) or whole blood coagulated in small-diameter Tygon tubing and or ePTFE grafts. The maximum purging pressures rose with increased clot maturity for fibrin glue, PRP, and whole blood in both Tygon and ePTFE tubes. Although the rapidly coagulating fibrin glue in nonpermeable Tygon tubing yielded highly consistent purging curves, the significantly longer and more variable clotting times of PRP and whole blood, and the porosity of ePTFE grafts, significantly diminished the consistency of the purging curves.

Keywords

vascular graft; thrombogenicity; test method; hemocompatibility; fibrin

INTRODUCTION

It is estimated that in 20% of the 500,000 coronary artery bypass grafting (CABG) surgeries performed annually in the United States, patients lack suitable autologous vessels for autografting due to the progression of atherosclerotic disease or due to prior surgeries.^{1–3} This fraction is expected to grow as both the incidence of repeat interventions and mean life spans of people increase.^{4,5} Unfortunately, no current synthetic small-diameter grafts are suitable for CABG because they fail with high frequency due to thrombosis and intimal hyperplasia—both of which give rise to resistance to blood flow. In general, the level of resistance to blood flow that leads to ischemic effects occurs well before complete vessel blockage.⁶

In vitro methods used to study thrombus formation in actual synthetic grafts have focused on endpoints such as graft patency, luminal coverage of adherent thrombus, amount and morphology of adhered platelets or amount of particular biomolecular species generated by the coagulation cascade.^{7–12} To our knowledge, no *in vitro* studies have measured the relationship between the extent of coagulation in the vessel lumen and the resultant resistance to flow.

Two primary physical changes occur within the vessel lumen during thrombus formation: cross-sectional area available for blood flow decreases and blood viscosity increases as the blood coagulates,^{13–16} both of which lead to increased resistance to blood flow. According to the electrical circuit analogy for Poiseuille's law, which assumes the ideal case of laminar flow of a Newtonian fluid through a tube, resistance to flow is given by the following equation:

$$R = \frac{8\mu L}{A_{\text{available}}^2} \quad (1)$$

where R is resistance to flow, μ is fluid viscosity, $A_{\text{available}}$ is the cross-sectional area available for flow (neglecting potential porous flow through clots for simplicity), and L is the length of the tube contacting the fluid.^{17,18} The resistance to flow in blood vessels is more commonly described as being patent (lumen unblocked) or occluded (lumen partially to completely blocked). Rearrangement of Eq. (1) expresses the relationship in terms of the cross-sectional area of the unblocked patent vessel (A_{patent}) and the cross-sectional area of the lumen occluded by thrombus (A_{occluded}), where the percent vessel occlusion is given by $100 \times (A_{\text{occluded}}/A_{\text{patent}})$.

$$R = \frac{8\mu L}{\left[A_{\text{patent}} \left(1 - \frac{A_{\text{occluded}}}{A_{\text{patent}}}\right)\right]^2} \quad (2)$$

To a first approximation, resistance to blood flow should be linearly proportional to blood viscosity and inversely proportional to the square of the cross-sectional area still available for flow.^{19,20} However, thrombus formation in a vascular graft imposes a number of nonidealities: (1) blood is non-Newtonian; (2) coagulating blood transitions from a watery liquid to a viscous fluid and ultimately to a stiffening viscoelastic clot; and (3) adherent thrombus introduces a nonuniform cross-sectional area along the length of a graft.

In the current study, a closed pressure system connected to small-diameter tubes [either 3 mm inner diameter (ID) synthetic vascular grafts or graft mimics] was used to measure the coagulation-induced buildup upstream pressure within the graft lumen (Figure 1). This buildup of “purging pressure” is an indicator of flow resistance as shown in Eq. (3), where Q is the average rate of fluid flow and R is the average resistance between sampled points of pressure upstream (P_{purging}) and downstream (atmospheric pressure, P_{atm}) of a luminal occlusion.

$$P_{\text{purging}} = Q \frac{8\mu L}{A_{\text{available}}^2} - P_{\text{atm}} = QR - P_{\text{atm}} \quad (3)$$

Grafts were loaded with fluid and allowed to cure for set times. The cured graft contents were then compressed at a constant rate until the graft contents were purged. The resultant buildup of pressure proximal to loaded grafts was measured continuously by pressure transducers. The greater the extent of coagulation in the graft lumen the higher was the pressure needed to purge the coagulum from the graft. The pressure just prior to purging (maximum purging pressure) served as an indicator of resistance to flow and was the primary endpoint for experiments.

The pressure system response was validated by purging a series of solutions of known viscosity from Tygon tubes with well-defined cross-sectional areas available for flow as economical and nonpermeable small-diameter conduits for initial system validation. The system was then applied to purging cured solutions of coagulating fibrin glue, platelet-rich plasma (PRP) and whole blood from small-diameter Tygon tubes and permeable ePTFE vascular grafts. To our knowledge, this work is the first demonstration of using upstream pressure buildup to quantify the physical interaction between the lumen of a vascular graft and a coagulating clot.

MATERIALS AND METHODS

All methods were performed at room temperature (approximately 20°C) unless otherwise noted.

Materials

Tygon laboratory tubing with ID of 3 mm and wall thickness of 1 mm (Saint-Gobain Performance Plastics, Akron, OH) was used as a mock graft material. The vascular grafts were tubes of expanded polytetrafluoroethylene (ePTFE) with 2.9 mm ID and 0.21 mm wall thickness (International Polymer Engineering, Tempe, AZ). Tygon and ePTFE tubes cut to 4 cm in length were sonicated in distilled water for 10 min, absolute ethanol for 10 min, water again for 10 min and then dried with nitrogen gas and in a 50°C oven for 1 h. The tubes were used within 2 h of cleaning.

Mixtures of pure glycerol (Sigma Aldrich, St. Louis, MO) and distilled water were used to generate 0, 50, 80, 90, 95, and 100% (v/v) glycerol solutions. This set of glycerin solutions was used for all experiments to minimize experimental variability.

Fibrin glue solutions were prepared by combining solutions of 12 mg/mL bovine fibrinogen (Sigma) and 0.25 U/mL bovine thrombin (Sigma), each in Dulbecco's phosphate-buffered saline (DPBS, pH 7.4, Gibco) supplemented with 1% (w/v) bovine serum albumin (Sigma).

Fractional occlusion of Tygon tubes

Bondable (etched) PTFE sheets (Small Parts, Logansport, IN) of thickness 0.03 inch were cut with a VLS6.60 laser system (Universal Laser, Scottsdale, AZ) into a series of annuli with an outer diameter of 7 mm and IDs of 2.683, 2.324, 1.897, 1.439, 0.949, 0.671, and 0.424 mm. After cutting out the annuli, vulcanized PTFE debris was gently removed with a hobby knife (X-ACTO, Columbus, OH) and digital calipers (Mitutoyo, Aurora, IL) were used to confirm IDs.

The annuli were fixed to the exit orifice of the 3 mm ID Tygon tubes, thus decreasing the cross-sectional area available for flow to 20, 40, 60, 77, 90, and 95% of its original value. A thin film of industrial superglue (Elmens Products, Columbus, OH) was used to center the annuli in the tube opening, followed by two additional applications of superglue at the annulus-tube interface.

Blood collection and plasma isolation

Approximately 150 mL of peripheral blood was drawn by venous puncture into K2 ethylenediaminetetraacetic acid tubes [Beckton Dickinson and Company (BD), Franklin Lakes, NJ] from a single healthy volunteer according to protocols approved by the Duke University institutional review board. To minimize clotting time (CT) variability, collected blood was pooled into a single container and then aliquoted into three 50 mL centrifuge tubes. Two of the blood-containing tubes were centrifuged at 205g for 20 min at 25°C to generate PRP. The partitioned top layers of PRP were pipetted off the top and combined into a single 50 mL centrifuge tube. Both whole blood and PRP were stored at room temperature and used within 36 h of isolation.

Fluid loading into ePTFE vascular grafts

The porosity of ePTFE allowed coagulating solutions to weep through the tubes, necessitating a particular procedure to load the solutions (Supporting Information Figure 1). Cleaned ePTFE tubes were pulled over the hose barbs (3.17 mm maximum diameter) of male luer-lock tubing adaptors (Harvard Apparatus, Holliston, MA) to form tight seals while leaving approximately 250 μ L tube volume beyond the barbs. Maintaining the tube/adapters in a horizontal orientation, clotting solutions were slowly injected into the tubes via syringes with the needles extending through the adaptors and just beyond the hose barbs. While still horizontally oriented, each tube end was crimped with a surgical ligating clip (Teleflex Medical, Research Triangle Park, NC) just beyond the location of the blood. Loaded tubes were then vertically oriented and “burped” to remove air bubbles by gently compressing the tubes along their lengths with gloved fingers. Care was taken not to alter the tube cylindrical geometry during this process. Tubes remained in a vertical orientation while clotting solutions cured by coupling the loaded tube/adaptor to a suspended luer-lock syringe.

Fibrin CTs

Fibrin glue CTs were determined in polypropylene 96-well plates (BD). Fibrin glue solutions were prepared as described above to have a final fibrinogen and thrombin concentrations of 6 mg/mL and 0.125 U/mL, respectively. These solutions were then immediately transferred to the well plates and allowed to cure for set durations. The CT was defined as the time at which a fibrin strand could be drawn from the solution with a pipette tip. A rough CT was determined first by testing solutions at 1 min curing intervals. A more precise CT was then determined by using shorter curing intervals centered around the approximate CT. This process was iterated until the CT was determined within 10 s.

Additional Supporting Information may be found in the online version of this article.

PRP and blood CTs

Many factors (blood-contacting surface type, surface area to-volume ratio, physical orientation, level of air exposure, etc.) impact the CT of blood and PRP. This required that blood and PRP CTs were measured using the same clotting conditions used in the pressure experiments. Blood and PRP were reactivated with 0.1 M CaCl_2 at a 1:10 ratio to blood or PRP. CTs in Tygon tubes were determined by drawing approximately 200 μL reactivated blood or PRP into cleaned tubes attached to 5 mL luer-lock syringes (BD). After blood or PRP were allowed to cure for various durations, tubes were then purged and the contents were examined for evidence of clotting. The blood CT was defined as the shortest curing duration that generated a thrombus. A rough CT were determined first by testing at 10 min curing intervals, followed by more precise CTs determined using shorter curing intervals centered around the rough CT. This process was iterated until the CT was determined within 1 min.

Rheology of glycerin solutions, fibrin glue and blood

Rheological measurements were performed with a cone and-plate Brookfield DV-III Rheometer outfitted with a LV spring and a CP40 spindle (Brookfield Engineering Laboratories, Stoughton, MA). The empty rheometer was zeroed with an idle torque reading of <1%, and the gap between the cone and plate was established. Volumes of 0.5 mL glycerin solution, fibrin glue, or reactivated blood were loaded, and the viscosity was measured over time using a low shear rate of 2.25 s^{-1} , which was chosen to minimize shear induced activation of blood. As blood approached its CT, the torque on the spring rapidly increased until the torque limit was reached and additional data could not be gathered. RHEOCALC software (Brookfield) was used to record and process raw data into values of viscosity.

Spectroscopic characterization of fibrin clotting reaction

Fibrin glue solutions were prepared as described above to have a final fibrinogen concentration of 6 mg/mL and final thrombin concentrations of 0.25, 0.125, 0.0625, and 0.03125 U/mL. These components were mixed, immediately transferred to polypropylene 96-well plates (BD), and absorbances were monitored over time at 405 nm in a μQuant microplate reader (BioTek, Winooski, VT). Absorbance data were baseline corrected, and the time axis was offset to correct for differences between experimental runs in the initiation of clotting and the beginning of data acquisition.

Pressure measurement system

Figure 1(A) shows the pressure measurement system consisting of a vertically oriented luer-lock 5 mL syringe secured in a programmable syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA), and connected to a 3-way T-fitting. One T-fitting connected the syringe to the Tygon tubing or ePTFE grafts. The other two T-fittings connected separately via pressure lines (6 cm lengths of 0.5mm ID low gas permeability PharMed BPT tubing, Saint-Gobain Performance Plastics) to a low-sensitivity/high-resolution pressure transducer (part MPXC2011DT1; FreeScale Semiconductor, Tempe, AZ) and a high-sensitivity/low-resolution pressure transducer (part MPX5100GP, FreeScale Semiconductor). Both

transducers were supplied with 5 V DC (CPS250 power supply; Tektronix, Beaverton, OR). The output of the high-resolution transducer was amplified 10003 and band-stop filtered (0.1–100Hz) by a differential amplifier (DAM 50; World Precision Instruments, Sarasota, FL) running in differential mode. Pressure signals were interfaced with a computer via a National Instruments 9215 compact data acquisition card in a Legacy CompactDAQ chassis (National Instruments, Austin, TX) that permitted simultaneous capture of the two independent pressure transducer signals without introducing crosstalk. Data were acquired using LabVIEW 2010 software (National Instruments). All junctions were connected within short Tygon tubes and hermetically sealed with industrial superglue.

The voltage–pressure relationship of the system was determined by assuming that the product of gas pressure and volume in a closed system is conserved according to Boyle’s law.²¹ The hose barb attached to the Tygon tubes was closed while maintaining an initial ambient pressure of 760 mmHg. Voltage data from the pressure transducers were recorded while compressing the system in increments of 2% of the known starting volume. Given these values of initial volume, initial pressure and compression levels, the pressure at each compression level was computed and plotted against the voltage signals from the transducers. Calibration curves were then generated for each transducer and used to convert experimental raw voltage data to pressure values.

Purging pressure measurements

Prior to making measurements, the syringe plunger was fully depressed to ensure a consistent and minimal dead space volume of the system. For Tygon tubing, cleaned tubes were pulled over the hose barb of the T-fitting such that approximately 220 μ L tube volume remained distal to the barb. The distal inlet of each graft was submerged into a glycerin solution, fibrin glue, PRP, or whole blood, and the syringe pump was used to draw up 200 μ L of fluid. Coagulating solutions were allowed to cure for specific durations based on previously determined CTs [Figure 1(A)]. Data acquisition was initiated 5 s prior to purging grafts to establish a baseline signal. After curing for the appropriate duration, the syringe pump was used to purge grafts at a constant flow rate of 2.4 mL/min. Due to the resistance to flow of the fluids within the grafts, trapped air proximal to the grafts was compressed, resulting in increased systemic pressure while purging [Figure 1(B)]. The accumulated pressure was released upon expelling sufficient fluid or coagulating material to create a path through which the compressed air could escape. The maximum purging pressure is the pressure achieved just prior to expelling the contents of the tubes.

Pressure measurements of ePTFE grafts were performed using the same procedure as above with the following modifications. A luer-lock type connector (Cole Parmer) allowed fluid-loaded ePTFE grafts to be screwed directly onto the T-fitting attached to the syringe. Just prior to reaching the curing duration of the coagulating solution, grafts were attached and the crimped end of each graft was removed to permit purging by cutting with surgical scissors just above the ligation clip. Purging and data acquisition were then carried out as in the protocol for Tygon grafts.

Acquired raw voltage data were baseline-corrected by subtracting the average voltage value recorded over 5 s while the system was idle before purging grafts. Voltages were then

converted to pressure values using the calibration curves generated for each transducer. Maximum pressures achieved while purging grafts were identified for both transducers. Pressure values obtained from the amplified higher resolution transducer were more precise and were thus used whenever possible. Above 60 mmHg, pressure values of the lower resolution transducer were used as the higher resolution signal became saturated.

Modeling theoretical flow resistances

Equation (1) was used to compute theoretical flow resistances from experimentally derived values of viscosity, cross-sectional area available for flow, and tube length. To model fibrin glue resistances, curing fibrin glue was assumed to be a Newtonian fluid with a given apparent viscosity at a particular curing time as determined by rheology. Computed resistances were then normalized to the largest calculated value and plotted.

Statistics

Differences between experimental conditions were determined using multivariate analysis of variance (ANOVA). The significance of individual differences was established using the post hoc Tukey's honestly significant difference test where appropriate. Significance was assumed at $p < 0.05$.

RESULTS

Solution viscosity and fractional occlusion pressure measurements

Figure 2 compares the maximum purging pressure accumulated in the Tygon tubing as a function of percent occlusion (area of exit orifice blocked by the annulus) for a range of fluid viscosities to theoretical flow resistance computed for the same conditions using Eq. (2). The maximum purging pressure increased with increased glycerin solution viscosity and with increased percent occlusion. Each fluid of a given viscosity exhibited a similarly shaped nonlinear increase in purging pressure with increased percent occlusion, with this increase being most pronounced for the most viscous fluids. Each viscosity or occlusion level was significantly different from all others ($p < 0.0001$ for most comparisons), except for the statistically similar viscosity levels of 1 and 8.3 cP ($p = 0.96$). Theoretical resistances followed similar trends to maximum purging pressures observed experimentally. In both cases, the blockage-induced pressure did not increase substantially until after 60% occlusion.

Fibrin glue and whole blood pressure measurements

Figure 3 shows the increasing pressure generated while purging fibrin glue, PRP and whole blood samples from Tygon tubes and ePTFE grafts as a function of curing duration. Note that the data in Figure 3 are presented as a fraction of CT that is defined as the duration required for gelation. This normalization was necessary to correct for slight variations in the CT for the different fibrin glue preparations (seconds) and more substantial variations in the CT of reactivated whole blood (minutes). Overall, both fibrin glue and whole blood exhibited the same behaviors in non porous Tygon tubes. The fibrin glue results yielded the most highly consistent curves for the same curing times [Figure 3(A)]; however, blood samples in Tygon tubes exhibited greater variability during the build up of purging pressures

for the same curing times [Figure 3(B)]. Consistent results were also achieved with fibrin glue in ePTFE grafts (individual traces not shown). PRP and whole blood cured in ePTFE grafts exhibited the most variable purging pressures, ranging from near baseline values to ~140 mmHg [Figure 3(C)] at gelation.

Fibrin glue samples cured for less than the CT [1.0 CT in Figure 3(A)] were purged spontaneously when the maximum purging pressure was reached; however, the maximum purging pressure plateaued for fibrin glue cured for longer than the CT before the gelled fibrin was ejected in both Tygon and ePTFE (only Tygon shown). The duration of this plateau increased the longer the glue was cured beyond the CT. This phenomenon also occurred with blood and PRP but was only consistently observed well beyond the CT (1.5 CT in Figure 3) in Tygon tubes. Inspection of the purged grafts at longer curing times showed that the outer portions of the coagulum adhered to lumen wall after the center portion of the coagulum was purged.

Figure 4 shows the effect of curing duration on maximum pressures generated upon purging fibrin glue and blood from Tygon tubes, and purging fibrin glue from ePTFE grafts. All three cases exhibited increased maximum purging pressures with increased curing times ($p < 0.0001$ for all comparisons except $p < 0.05$ for 25 vs. 50% greater than the CT). The inset in Figure 4 shows that these higher pressures correlated with increased turbidity of curing fibrin glue solutions. Both whole blood and fibrin glue exhibited significantly higher maximum purging pressures with increasing curing times ($p < 0.0001$ for most comparisons). However, maximum purging pressures for whole blood were much more variable near the CT (~30 min), which was substantially longer than the CT of fibrin glue in Tygon (~4 min). Consequently, statistically similar pressures were observed for blood and fibrin glue for all curing durations. Much lower variability (similar to fibrin glue) was produced from purging blood cured for less than or 50% longer than the CT.

The CTs of whole blood and PRP in ePTFE grafts (~120 min) were also much longer than that of fibrin glue in ePTFE grafts (~4 min). Maximum purging pressures produced by the clotting of whole blood and PRP in ePTFE grafts are shown in Figure 5. Purging whole blood and PRP cured until their respective CTs resulted in significantly greater maximum pressures than purging these clotting solutions just after reactivation with calcium ($p < 0.0001$). High variability was observed in purging blood or PRP at the CT, with maximum purging pressures ranging from near baseline to almost 140 mmHg. As a result, maximum pressure values for purging whole blood and PRP both before and at the CT were statistically similar despite a large number of trials ($n = 10$).

Rheology of fibrin glue

The apparent viscosity of fibrin glue increased with curing time [Figure 6(A)] and was found to increase more slowly than did the maximum purging pressure over the same range of curing times [Figure 6 (B)]. While apparent viscosity increased and seemed to approach an asymptotic value from curing times of 90–150% of the CT, substantially higher variability at longer curing durations resulted in statistically similar apparent viscosity values at and above the CT.

Comparison of experimental results to theory

Figure 7 illustrates how experimentally measured maximum purging pressures deviated from theoretical pressures calculated from Eq. (1) for both pure glycerin and fibrin glue. Measured pressures and theoretical resistances both increased with increasing tube occlusion and apparent viscosity, but maximum pressures generated by purging glycerin and fibrin glue were underpredicted and overpredicted, respectively, by theory.

DISCUSSION

The purging pressure measurement system described here was capable of precisely detecting physiologically relevant changes in solution viscosity or the cross-sectional area available for flow. Fibrin glue and blood coagulation in Tygon and ePTFE grafts were also precisely monitored, with more developed clots resulting in greater purging pressures and pressures induced by the presence of blood clots being generally more variable than those of fibrin clots.

The range of purging pressures measured in these studies required the simultaneous use of two pressure transducers to expand the dynamic range of the system and to improve low-end sensitivity. As this dual transducer configuration was capable of highly reproducible measurements with generally tight error, experimental variability that was observed in more complex, less well-defined sample measurements such as fibrin glue or blood could be attributed to the intrinsically high variability of these biological solutions and not the pressure system itself.

The response of the pressure measurement system to the range of physiologically relevant fluid viscosities and percent occlusions was reproduced qualitatively by Poiseuille's law [Eq. (1)]. However, regression plots revealed this law to underestimate the measured pressure increases stemming from increased viscosity and percent occlusion [Figures 2(B) and 7; regression plots not shown]. We attribute the deviation of our experimental measurements from Poiseuille's law (which describes laminar flow in a tube of consistent ID) primarily to the abrupt decrease diameter at the terminus of the Tygon tubes. Other deviations from ideal behavior, such as energy losses due to unaccounted for frictional factors, likely also contributed to experimentally observed maximum purging pressures being underpredicted by computed theoretical values. Similar to the clinical observation that coronary artery blockage is asymptomatic until around 60–70% occlusion, (private communication²²) these results show that both maximum purging pressures and computed flow resistances did not become substantial until tube blockages of 60% or higher.

Fibrin glue with its well-defined reaction kinetics was chosen as a blood clot mimic in the initial testing of clotting solutions with the system. These initial tests were performed using Tygon tubing as economical and nonpermeable conduits that possess fluorinated surface chemistries similar to ePTFE grafts. Fibrin glue is a buffered solution containing fibrinogen and thrombin that react to generate an a cellular interpenetrating network of fibrin strands.^{19,23} Importantly, the particular formulation of fibrin glue used in this study did not produce cross-linked clots as it lacked factor XIII.^{24,25} This highly simplified clotting scheme facilitated the systematic exploration of changes in flow resistance resulting from

curing a clotting solution for various durations. In contrast, blood clotting occurs in the context of an enormously complex biological milieu involving erythrocytes, platelets and many additional serum protein components in the coagulation cascade that are absent from fibrin glue. In particular, platelets and erythrocytes are incorporated into and strengthen the fibrin clot by directly binding with and being entrapped by fibrin.^{26,27} The presence of factor XIII in blood also permits the covalent cross-linking of fibrin chains, reinforcing the clot and increasing its bulk strength.^{28,29} Note that two distinctly characteristic clot types, platelet-rich “white clots” and fibrin-rich “red clots” (so-named as they readily entrap erythrocytes), preferentially form under arterial and venous flow conditions, respectively. Blood clots in this study were formed under static conditions and thus likely differed from either of these clot types, but blood coagulum still offered a significantly different alternative clotting material to fibrin glue while possessing much more similar character to luminal clots formed *in vivo*.

As fibrin glue cures in the graft lumen, fibrin strands thicken, lengthen and intertwine, increasing clot strength and wall adhesion.^{19,30,31} Blood clots are additionally strengthened over time by the incorporation of platelets and cells into the crosslinking fibrin. We believe that these factors gave rise to (1) stronger resistance to flow at longer curing durations and therefore higher maximum purging pressures and (2) the plateauing of the maximum purging pressure for the most coagulated samples (Figures 3 and Figure 4). However, the inset in Figure 4 shows that the turbidity spectra of curing fibrin glue continued to increase linearly even when the maximum purging pressures did not increase with longer curing durations. The fibrin therefore continued to polymerize substantially even though the strength of the coagulum appeared to no longer increase.

Despite substantial physical and biological differences between fibrin glue and blood coagulum, maximum purging pressures generated at corresponding degrees of coagulation were statistically similar in Tygon grafts (Figure 4). The statistically similar data suggest that the fibrin component of the blood coagulum plays a dominant role in the resistance to flow over the probed range of curing times. However, it is possible that the maximum level of system compression was insufficient to elicit observable differences at 50% beyond the CT. Should differences in maximum purging pressures exist, they may become apparent at higher levels of system compression and/or curing times longer than those tested here.

It should be noted that Tygon tubing is not a vascular graft material, fibrin glue coagulum does not form in the same manner as blood clots, and that static testing does not account for the variety of dynamic flow and environmental conditions that one encounters *in vivo*. However, our data point to substantially different clot types offering similar resistance to flow when obstructing both mimicked and actual vascular graft materials. That said, virtually all *in vitro* assays used to assess blood-material interactions, such as whole blood clotting time are designed to be simple and reliable measurement schemes that generate comparative results. The current study suggests that the devised system is potentially capable of assessing a wide range of physiologically relevant clotting conditions in vascular graft materials.

Pressure measurements made using blood and PRP proved to be more difficult. Even though the pressure system was capable of precise measurements and precautions were taken to minimize variability, the highly variable results obtained for blood and plasma clotting reveal the notoriously difficult nature of making quantitative measurements with blood and PRP near the CT [Figures 3(B,C)–5]. Under identical testing conditions, the clotting kinetics for a given blood sample were primarily dependent on the degree of blood activation (i.e., extent of coagulation cascade activation; also applies to PRP).^{32,33} Indeed, the same blood sample was observed to clot nearly four times more slowly in ePTFE (~120 min) than in Tygon (~30 min)—an effect that was corrected for by normalizing experimental curing times to permit direct comparisons between clotting agents with different CTs. However, this effect should not have contributed to the observed variability in pressure data for these clotting solutions as presented data corresponded to testing within the same graft type. Therefore a number of factors could have contributed to the variability of this data: (1) inconsistency in blood handling, which would have activated clotting pathways to different extents, (2) decreased viability of platelets and erythrocytes (for whole blood) as collected blood and PRP aged, causing a drift in the CT, and (3) differential reactivation of blood and PRP due to having added back unequal amounts of calcium. It is also possible that the carefully determined CT of blood drifted over the course of the experiments, resulting in clots of differing maturity at the same curing time.

The largest such variability was observed for blood and plasma clotting in ePTFE grafts. The increased specimen handling when loading the grafts with blood may have activated PRP or blood to different extents. Increased variability also stemmed from the spatial variation of blood and PRP clotting rates, with the fastest clotting occurring proximally in the graft. The relatively long CT of blood and PRP (around 2 h) may have allowed (1) some fluid to weep out of the porous material over time and (2) the formed elements of the blood to sediment, enriching the distal ends of the grafts in cells/platelets and leaving mostly plasma in the proximal end to result in inhomogeneous clots.³⁴ Consistent results were achieved for fibrin glue in ePTFE as its relatively fast gelation kinetics circumvented these issues. A method for loading porous grafts and reducing blood partitioning that minimized specimen handling could mitigate variability and allow meaningful systematic studies to be performed in porous graft materials.

As fibrin glue cures and transitions from a fluid to a stiffening viscoelastic gel, the same biophysical phenomena that led to increased bulk coagulum strength also caused the observed increase in apparent viscosity over time toward an asymptotic value [Figure 6 (A)]. However, despite also approaching a terminal value as clotting proceeded, maximum purging pressures appeared to increase to a greater degree than apparent viscosity at later curing times [Figure 6(B)]. This discrepancy may have been an artifact caused by the shearing of maturing fibrin clots during the rheological measurements, possibly breaking apart growing fibrin strands, and therefore underestimating apparent viscosity measurements at later curing times.

Finally, theoretical resistances to purging fibrin glue computed using the rheologically determined apparent viscosities were substantially higher than were the corresponding maximum purging pressures (Figure 7). One possibility is that theoretical resistances

assumed that the clot structure and apparent viscosities were homogenous throughout the clot, but actual fibrin clots were nonuniform and were locally weaker, particularly in the center region, which may have failed preferentially at lower maximum purging pressures.

CONCLUSIONS

The goal of this study was to quantitatively assess the maximum flow-induced pressure upstream of curing blood coagulum within the lumens of synthetic vascular grafts. By measuring pressure accumulation, the devised system was able to provide a quantitative indicator of the flow resistance that developed during the coagulation of fibrin glue, PRP, and whole blood within the lumens of Tygon tubes and ePTFE vascular grafts. The coagulation of fibrin glue and whole blood both showed a maximum purging pressure that increased with curing time. The fibrin glue data, being the best behaved of the two coagulums, yielded similar results in both nonpermeable Tygon tubes and permeable ePTFE grafts. Whole blood, being more difficult to work with, also performed similarly in the Tygon tubes but both the permeability of the ePTFE grafts and relatively long and variable CTs proved to be problematic for blood and plasma studies. This approach provided insight into how blood flow resistance is influenced by a number of clinically relevant factors, such as the level of vessel occlusion and the physical nature of the resident coagulum. While the described system could only quantify resistance as a lumped parameter, future refinement of this technique may enable the specific clot failure mechanism (i.e., shearing from lumen or rupture) to be elucidated, and thus provide an *in vitro* predictive test of graft–thrombus interactions.

Acknowledgments

Contract grant sponsor: National Institutes of Health (NIH); contract grant number: R01HL-44972

The authors would like to thank Matthew Brown and Dr. Marcus Henderson for use and set-up of the power supply, amplifier and DAQ card. They also thank Dr. Bruce Klitzman for insightful discussions.

REFERENCES

1. Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, et al. Writing Group Members. Heart disease and stroke statistics— 2010 update: A report from the American Heart Association. *Circulation*. 2010; 121:e46–e215. [PubMed: 20019324]
2. Grossman DK, Middlebrook DA. Thoratec laboratories submits data to begin Canadian clinical trials for Aria coronary artery bypass (CAB) graft. Thoratec's Heart Beat Newslett. 1999;13.
3. Cagiannos C, Abul-Khoudoud OR, DeRijk W, Shell DH, Jennings LK, Tolley EA, et al. Rapamycin-coated expanded polytetrafluoroethylene bypass grafts exhibit decreased anastomotic neointimal hyperplasia in a porcine model. *J Vasc Surg*. 2005; 42:980–987. [PubMed: 16275457]
4. Weintraub WS, Jones EL, Graver JM, Guyton RA. Frequency of repeat coronary bypass or coronary angioplasty after coronary artery bypass surgery using saphenous venous grafts. *Am J Cardiol*. 1994; 73:103–112. [PubMed: 8296729]
5. Oeppen J, Vaupel JW. Broken limits to life expectancy. *Science*. 2002; 296:1029–1031. [PubMed: 12004104]
6. Chierchia S, Brunelli C, Simonetti I, Lazzari M, Maseri A. Sequence of events in angina at rest: Primary reduction in coronary flow. *Circulation*. 1980; 61:759–768. [PubMed: 7357718]

7. Hamlin GW, Rajah SM, Crow MJ, Kester RC. Evaluation of the thrombogenic potential of three types of arterial graft studied in an artificial circulation. *Br J Surg.* 1978; 65:272–276. [PubMed: 147719]
8. Heyligers JMM, Verhagen HJM, Rotmans JI, Weeterings C, de Groot PG, Moll FL, et al. Heparin immobilization reduces thrombogenicity of small-caliber expanded polytetrafluoroethylene grafts. *J Vasc Surg.* 2006; 43:587–591. [PubMed: 16520178]
9. Fleser PS, Nuthakki VK, Malinzak LE, Callahan RE, Seymour ML, Reynolds MM, et al. Nitric oxide-releasing biopolymers inhibit thrombus formation in a sheep model of arteriovenous bridge grafts. *J Vasc Surg.* 2004; 40:803–811.
10. Williams SK, Rose DG, Jarrell BE. Microvascular endothelial-cell sodding of eptfe vascular grafts —Improved patency and stability of the cellular lining. *J Biomed Mater Res.* 1994; 28:203–212. [PubMed: 8207032]
11. Mannucci PM. Mechanisms, markers and management of coagulation activation. *Br Med Bull.* 1994; 50:851–870. [PubMed: 7804735]
12. Tripodi A, Mannucci P. Markers of activated coagulation and their usefulness in the clinical laboratory. *Clin Chem.* 1996; 42:664–669. [PubMed: 8653889]
13. Kaibara M. Rheology of blood coagulation. *Biorheology.* 1996; 33:101–117. [PubMed: 8679959]
14. Dintenfass L. Viscosity and clotting of blood in venous thrombosis and coronary occlusions. *Circ Res.* 1964; 14:1–16. [PubMed: 14106548]
15. Davies MJ, Thomas A. Thrombosis and acute coronary-artery lesions in sudden cardiac ischemic death. *N Engl J Med.* 1984; 310:1137–1140. [PubMed: 6709008]
16. Farb A, Burke AP, Tang AL, Liang Y, Mannan P, Smialek J, et al. Coronary plaque erosion without rupture into a lipid core : A frequent cause of coronary thrombosis in sudden coronary death. *Circulation.* 1996; 93:1354–1363. [PubMed: 8641024]
17. Barnes RW. Hemodynamics for the vascular surgeon. *Arch Surg.* 1980:115–216.
18. Naeije R. Pulmonary vascular resistance. *Intensive care Med.* 2003; 29:526–529. [PubMed: 12800831]
19. Ryan EA, Mockros LF, Weisel JW, Lorand L. Structural origins of fibrin clot rheology. *Biophys J.* 1999; 77:2813–2826. [PubMed: 10545379]
20. Weisel JW. The mechanical properties of fibrin for basic scientists and clinicians. *Biophys Chem.* 2004; 112:267–276. [PubMed: 15572258]
21. Levine, IN. *Physical Chemistry.* New York, NY: McGraw-Hill; 2008.
22. Povsic, TJ. Personal Communication to Nichols. Durham, NC: M.D. Division of Cardiology, Duke University Medical Center; 2011.
23. Wolberg AS. Thrombin generation and fibrin clot structure. *Blood Rev.* 2007; 21:131–142. [PubMed: 17208341]
24. Pisano JJ, Finlayson JS, Peyton MP. Chemical enzymic detection of protein cross-links Measurement of epsilon-(gamma-glutamyl)lysine in fibrin polymerized by factor XIII. *Biochemistry.* 1969; 8:871–876. [PubMed: 4976409]
25. Lorand L, Rule NG, Ong HH, Furlanetto R, Jacobsen A, Downey J, et al. Amine specificity in transpeptidation Inhibition of fibrin cross-linking. *Biochemistry.* 1968; 7:1214–1223. [PubMed: 5690562]
26. Lominadze D, Dean WL. Involvement of fibrinogen specific binding in erythrocyte aggregation. *FEBS Lett.* 2002; 517:41–44. [PubMed: 12062406]
27. Packham MA. Role of platelets in thrombosis and hemostasis. *Can J Physiol Pharmacol.* 1994; 72:278–284. [PubMed: 8069774]
28. Shen L, Lorand L. Contribution of fibrin stabilization to clot strength Supplementation of factor XIII-deficient plasma with the purified zymogen. *J Clin Invest.* 1983:71–1336.
29. Nielsen VG, Gurley WQ, Burch TM. The impact of factor XIII on coagulation kinetics and clot strength determined by thrombelastography. *Anesth Analg.* 2004:99–120.
30. Kjaergard H, Velada J, Pedersen J, Fleron H, Hollingsbee D. Comparative kinetics of polymerisation of three fibrin sealants and influence on timing of tissue adhesion. *Thromb Res.* 2000; 98:221–228. [PubMed: 10713324]

31. Glidden PF, Malaska C, Herring SW. Thromboelastograph assay for measuring the mechanical strength of fibrin sealant clots. *Clin Appl Thromb/Hemost*. 2000; 6:226–233.
32. Hattersley PG. Activated coagulation time of whole blood. *JAMA*. 1966:196–436.
33. Gravlee GP, Case LD, Angert KC, Rogers AT, Miller GS. Variability of the activated coagulation time. *Anesth Analg*. 1988:67–469.
34. Riha P, Liao F, Stoltz J. The effect of rouleaux formation on blood coagulation. *Clin Hemorheol*. 1997; 17:341–346.

FIGURE 1.

Method used to make measurements with the devised system. (A) Air pressure was monitored proximal to tested vascular grafts via two types of pressure sensors to provide sensitive measurements over a larger dynamic range. Tygon grafts were attached to the vertically oriented system and solutions were drawn into mock grafts using a syringe pump. Alternatively, ePTFE grafts were manually loaded with solution and attached to the system (not shown). After allowing the solution to cure for an appropriate duration, graft contents were purged at a constant flow rate. (B) Resistance to flow while purging results in compression of air proximal to the graft until a maximum pressure is reached. This maximum pressure served as the primary end point for pressure measurements.

A) Experimental Results

B) Theoretical Calculations

FIGURE 2.

Experimentally observed maximum purging pressures for a range of fluid viscosities and cross-sectional areas available for flow exhibited similar trends to theoretical flow resistances predicted by the electrical circuit analogy for Poiseuille's law. (A) Greater values of solution viscosity and fractional occlusion resulted in greater maximum pressures while purging Tygon grafts. Glycerin solutions ranging in viscosity from 1 to 1410 cP were purged from modified Tygon grafts with a range of percent occlusions, with each occlusion level significantly different from all others ($p < 0.0001$ except 40 vs. 60% at $p < 0.05$). More viscous solutions also generated significantly maximum higher pressures, with each tested viscosity significantly different from all others ($p < 0.0001$) except for 1 vs. 8.3 cP, which were statistically similar ($p = 0.96$). The inset provides a clearer view of the same data for fractional occlusions 60%. Data are mean $\pm$ standard error of the mean (SEM); $n = 3$ and small error bars are obscured by data points. (B) Theoretical flow resistances computed for corresponding experimental conditions possessed similar trends to measured data.

FIGURE 3.

Maximum pressures were (1) maintained for longer upon purging nonporous Tygon grafts of fibrin glue or whole blood cured for longer durations [fractions of clotting time (CT) as noted] and (2) highly variable at the same curing duration upon purging blood and PRP from porous ePTFE grafts. Individual pressure traces (representative of multiple trials) generated while purging fibrin glue (A) and whole blood (B) from Tygon reveal that both the maximum pressure and the period for which that pressure persisted increased with greater curing times. While fibrin glue pressure traces were highly reproducible, blood traces were highly variable in Tygon at and just after the clotting time (two traces of 1.0 and 1.25 CT shown to illustrate variability). (C) Purging whole blood and PRP cured for their clotting times (1.0 CT) from ePTFE was even more variable, with purging pressures ranging from near baseline to ~140 mmHg (three traces of each clot type shown to illustrate variability). Purging blood cured in Tygon for 150% of the clotting time (1.5 CT; ~45 min) (B) and in one case PRP cured in ePTFE for 100% of the clotting time (run 3 of 1.0 CT; ~120 min) (C) resulted in maintenance of maximum pressure until data collection stopped (data not shown).

FIGURE 4.

Higher maximum pressures resulted from purging fibrin glue and reactivated whole blood cured for longer durations from both Tygon and ePTFE grafts. Fibrin glue was allowed to cure for various durations normalized to its clotting time and then purged from both graft types, and same was done with blood in Tygon tubes. Curing of fibrin glue was also monitored spectroscopically (inset). Maximum pressures and solution turbidity (absorbance at 405 nm) both increased with increasing curing durations, with each curing duration significantly different from all others ($p < 0.05$ for *, **, ***, and ****). However, no significant differences between the type of graft or clotting solution were observed for a given curing duration. Data are mean $\pm$ SEM; $n = 4$.

FIGURE 5.

Highly variable maximum pressures were generated upon purging blood or PRP from ePTFE grafts at the clotting time. Reactivated blood and PRP were allowed to cure until the clotting time and then purged from ePTFE grafts. The clotting solutions were also purged just after reactivation for comparison. Maximum pressures generated from purging both whole blood and PRP were extremely variable, ranging from baseline pressure levels to nearly 140 mmHg [also shown in Figure 3(C)]. Consequently, maximum pressures generated by purging blood and PRP at each during duration were statistically similar ($p = 0.71$). Despite this variability, maximum pressures generated at the clotting time were significantly higher ($p < 0.0001$ for *) than those produced just after reactivation of blood or PRP. Data are mean $\pm$ SD; $n = 10$.

FIGURE 6.

The apparent viscosity of fibrin glue increased and became more variable as it cured up to and beyond its clotting time. (A) Beyond the clotting time, apparent viscosity increased more slowly and seemed to approach a plateau. Due to the high variability in measurements beyond the clotting time (>1.0), all apparent viscosities are statistically similar except for a significantly lower value at a curing time of 0.9 ($p < 0.05$ for *). Data are mean $\pm$ SEM; $n = 3$. (B) Maximum purging pressures increased faster and were less variable than apparent viscosity for corresponding curing times of fibrin glue (noted for each data point as fractions of clotting time). Statistics of pressure and apparent viscosity data are identical to those presented in Figures 3 and 6(A), respectively, and are omitted here for clarity. Viscosity data are mean $\pm$ SEM, $n = 3$, and pressure data are mean $\pm$ SEM, $n = 4$.

FIGURE 7.

Comparison of experimental and theoretical resistances generated by purging pure glycerin (gray line) and fibrin glue (black line) from Tygon tubes. The diagonal dashed line indicates perfect correlation of tube pressure described by Eq. (1) and the experimentally determined maximum purging pressures. Maximum purging pressure increased with percent tube occlusion and fibrin glue viscosity as theoretically predicted; however, maximum pressures generated when purging pure glycerin from the range of percent occluded tubes (Figure 2) were higher than predicted by theory, whereas maximum pressures generated when purging fibrin with a range of experimentally measured apparent viscosities from tubes [Figure 6(B)] were lower than predicted by theory.