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Evaluating the Effect of Shear Stress on Graft-to Zwitterionic PolyCarboxybetaine Coating Stability Using a Flow Cell

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Abstract:

Blood-contacting devices coated with anti-clotting materials would typically fail due to clot formation after about 2 weeks of exposure to blood flow. Our overarching hypothesis for their short-term success is that the failure modes of these anti-clotting coatings are either due to 1) a slowed-pace procoagulant protein fouling, 2) their erosion due to shear stress, or 3) a combination of both. This study however partly tests the hypothesis by evaluating the effect of shear stress on coating stability. This was done by exposing DOPA-PCB-300/dopamine coated polydimethylsiloxane (PDMS) to physiological shear stresses using a recirculation system which consisted of dual chamber acrylic flow cells, tygon tubing, flow probe and meter, and perfusion pumps. The effect of shear stress induced by phosphate buffered saline flow on coating stability was characterized with differences in fibrinogen adsorption between control (coated PDMS not loaded with shear stress) and coated samples loaded with various shear stresses. Fibrinogen adsorption data showed that relative adsorption on coated PDMS that weren't exposed to shear (5.73% ± 1.97%) was significantly lower than uncoated PDMS (100%, p < 0.001). Furthermore, this fouling level, although lower, was not significantly different from coated PDMS membranes that were exposed to 1 dynes/cm² (9.55% ± 0.09%, p = 0.23), 6 dynes/cm² (15.92% ± 10.88%, p = 0.14), and 10 dynes/cm² (21.62% ± 13.68%, p = 0.08). Our results show that DOPA-PCB-300/dopamine coating are stable, with minimal erosion, under shear stresses tested.
Introduction:

Large quantities of blood-contacting medical devices are used annually world-wide.\textsuperscript{1, 2} It is estimated that more than 200 million of these devices are utilized in patients in the U.S alone.\textsuperscript{3} They range from devices with small surface areas like catheters, vascular grafts, heart valves, cannulas, glucose, lactate sensors, and stents to those with moderate surface areas like pacemakers, artificial kidneys, and left ventricular assist devices. Then there are those with relatively larger surface areas like the artificial lungs, artificial hearts, and extracorporeal membrane oxygenation circuits.

The surfaces of these devices are made up of artificial materials that are different from endothelial cell surfaces, which interface with flowing blood.\textsuperscript{4–6} These cells express enzymes and secrete nitric oxide that maintain blood tone.\textsuperscript{7–10} Without these properties, blood rapidly activates into clots upon contact with artificial materials.\textsuperscript{11, 12} For blood-contacting devices, clot formation can cause cessation of blood flow and lead to device failure\textsuperscript{13–15}. Moreover, devices that do not fail may release clots into systemic circulation and cause embolic complications.\textsuperscript{14, 16–18} In life support devices these clots can result in morbidity and mortality. For instance, a small bore vascular graft serving as a coronary artery may occlude from formation and cause myocardial infarction (heart attack). With artificial lungs, clotting is especially problematic as they have relatively large surface areas (1.3-2 $m^2$) and a period of usage lasting from several weeks to months however they typically fail after 7-14 days with accompanying hemorrhagic complications.\textsuperscript{13–15, 18} Catheters, on the other hand, have a limited lifespan and do not reliably allow repeated sampling of blood or continuous pressure monitoring in patients as their small lumen diameters make them more prone to failure by clots\textsuperscript{19–22}. 
Current approaches for controlling biomaterial-induced clot formation have been largely inadequate. Commercial coatings have only shown moderate inhibition of clot formation in short-term studies\(^{23-32}\) and are not sufficient to allow large decreases in systemic anticoagulation. The most successful approach to date has been to chemically immobilize heparin on blood-contacting surfaces to reduce thrombosis and lower anticoagulant administration.\(^{33,34}\) Although this approach has been widely adopted, major limitations persist because the surface-bound heparin leaches, resulting in a progressive loss of anticoagulation activity.\(^{35,36}\) Other hydrophilic coatings including PHISIO (Sorin)\(^ {39}\), Trillium (Medtronic)\(^ {40}\), poly-2-methoxyethyl acrylate (PMEA) polymer\(^ {41}\) and sulfobetaine\(^ {42}\) that have undergone extensive human clinical evaluation have shown no drastic non-thrombogenic benefit compared to existing heparin-coated materials.\(^ {43,44}\) Systemic anticoagulants hence remain the adjunctive therapy of choice although they pose an increased risk of bleeding complications.\(^ {45-47}\)

Important factors that affect the efficiency these coatings include their stability and coverage on devices and mechanism(s) of inhibiting coagulation. Coatings that become unstable and erode against fluid shear progressively lose their anticoagulation activity and imperfections in coating can weaken the anti-coagulation effectiveness, as procoagulant proteins can adsorb at uncoated spots. Our overall hypothesis for their short-term success is that the failure modes of these anti-clotting coatings are either due to 1) a slowed-pace procoagulant protein fouling, 2) their erosion due to shear stress, or 3) a combination of both. To test our hypothesis, a relatively new coating material that has shown ultra-low auto-adsorption of pro-coagulant proteins, polycarboxybetaine,\(^ {48-50}\) was used to study the effect of shear stress on coating stability.
PDMS coated with DOPA-PCB-300/dopamine were exposed to shear stresses similar to those found in the vena cava, large veins, and conduit arteries.\textsuperscript{51}

**Methods**

**Flow Cell and Flow Recirculation Circuit Design:** The dual chamber flow cell and recirculation system design is shown in Figure 1. It consists of an acrylic (Custom Creative Plastics, FL) flow cell that was designed using Autodesk inventor (San Rafael, CA), a 3/16” I.D. and 5/16” O.D. tygon tubing circuit (Fisher Scientific, MA), a pump (Stöckert Shirley multflow roller blood perfusion pump, SOMA Tech. Bloomfield, CT), Transonic flow probe and meter (Transonic Inc. Cambridge MA) and Leuer lock priming ports. The flow cell assembly of top and bottom mates measures Length = 11.43 cm, width = 5.87 cm, height = 1.60 cm. and are attached to a recirculation circuit using polycarbonate connectors (Qosina, NY). The flow cell chamber measures a 1 cm x 6.35 cm x 0.5 cm with a hydraulic diameter = 0.67 cm, entrance area = 0.37 cm\(^2\). Test samples (surface modified or unmodified), represented by the rectangular piece inside the flow cell, are first affixed onto the bottom mate’s flow chamber already lined with an
adhesive nitrile sponge rubber gasket (Grainger.com, Lake Forest, IL) followed by the application of a gasket-lined top mate and compression of top and bottom mates with screws.

**Leak Testing, Pump Calibration, and Shear Stress Characterization:** To ensure no leakages during application of flows over samples, a leak test was performed under experimental test conditions. A PDMS sample measuring 8.89 cm x 2.54 cm was inserted into the recirculation circuit and primed with 35mL of phosphate buffered saline (PBS) ensuring that no air bubbles were present in the circuit before flow initiation. Recirculation was maintained for 8 hours at low flow (30 mL/min) and high flow (1500 mL/min). Since the blood perfusion pumps used in this experiment are roller pumps that display only digital revolutions per minute (rpm) readouts, it was necessary to determine their flow rates as a function of rpms. First, their tubing occlusion were set at the recommended clinical pump occlusion setting where a 100cm fluid column drops 25cm/min\(^{52-53}\). At this occlusion setting, a calibration curve of rpm versus flow rates was generated by pumping of PBS from a reservoir to an empty container. Rpm were set at 50, 100, and 150 and the pumped volume and pumping time recorded. An rpm to flow rate calibration curve was generated for each pump so that a relation of wall shear stress as a function average flow velocity (flow rate/cross sectional area), fluid dynamic viscosity, and hydraulic diameter of flow chamber could be developed.

Wall shear stress was calculated as

\[
\tau_o = f(\rho \times V_{avg}^2), \text{ where}
\]

\(\tau_o\) Wall shear stress (N/m\(^2\)).
Darcy-Wiesbach friction factor for the acrylic chamber surface is \( f = \frac{64}{Re} \) since flow is laminar,

- Reynolds number, 
- Density of fluid (\( kg/m^3 \)), 
- \( V_{avg} = \frac{flow\ rate}{flow\ chamber\ x-section\ area\ (m/s)} \).

The entrance length, \( L_e \), was expressed in terms of Reynolds number and hydraulic diameter as \( L_e = 0.06 \times Re \times D_h \) where \( D_h \) is the hydraulic diameter of the flow chamber given as \( \frac{4 \times A_c}{2(b+h)} \), where \( A_c \) is cross sectional area and \( b \) and \( h \) are width and height of the chamber entrance. The entrance length was calculated to be 0.04 cm using \( D_h = 0.67 \) cm and \( Re \sim 1 \).

**Coating PDMS with DOPA-pCB-300/dopamine and Coating Stability**

**Characterization:** PDMS membrane (NuSil Tech. CA) measuring 8.89 cm x 2.54 cm were casted via two-part polymerization process. Cured PDMS membranes were coated with DOPA-pCB-300 using a dip-coating process previously described.\(^{50}\) Briefly, PDMS was immersed in TRIS buffer (pH 8.5) containing dissolved 2.8 mg/mL DOPA-pCB-300/dopamine mixture at a ratio of 1:40. Buffer with PDMS was gently agitated for 2hrs. A schematic of the coating process is presented in [Figure 2].
Uncoated (N=5 samples at stagnant, no flow) and coated membranes (N=5 samples/test condition) were inserted into flow cells (Figure 3D) and the circuits were primed with phosphate buffered saline, pH= 7, (Sigma Aldrich, MO). Flows were initiated at 60 mL/min, 150 mL/min and 230 mL/min and recirculated through the flow cells for 8 hours. For each run, a set of four test conditions (coated PDMS with 0, 1, 6, and 10 dynes/cm²) were evaluated followed by test runs for uncoated no flow samples. These flows yield physiologically relevant shear stress of 1 dyn/cm², 6 dyn/cm² and 10 dyn/cm². The membranes were carefully removed after recirculation and stored in PBS. Three 1 cm x 1 cm pieces from each sample were sectioned and prepared for standard fibrinogen adsorption ELISA as previously described. The circuits were soaked in 10% bleach overnight, rinsed with DI water and dried with pressurized nitrogen between test runs.

Fibrinogen Adsorption Assay: Briefly, the 1 cm x 1 cm squares were placed into a 24-well plate and incubated in 1 mL of 1 mg/mL fibrinogen for 90 minutes at room temperature. The disks are then washed five times with PBS and incubated with 1mL of 1 mg/mL BSA (Sigma Aldrich) for 90 minutes at room temperature. The samples were again washed five times with PBS. Next, the samples were transferred into new wells and incubated in 1:1000 dilution of HRP (Sigma Aldrich) anti fibrinogen in PBS for 30 minutes, followed by another wash in PBS. The samples were then transferred to a new set of wells. The solution is then incubated in 500 uL of 1 mg/mL OPD (Sigma Aldrich) in 0.1 M citrate-phosphate buffer containing 0.03% hydrogen peroxide. This reaction was then quenched after 30 minutes by the addition of 500 uL of 1N HCL (Sigma Aldrich). The supernatant was then removed from each sample and transferred into
cuvettes. The absorbance of each supernatant was then measured at 492 nm using UV-vis spectrophotometer (Beckman Coulter, CT). It was expected that uncoated PDMS samples would have higher absorption of fibrinogen and thus higher UV-vis absorbance levels. The effect of coating erosion on biocompatibility was determined as the percent increase in fibrinogen adsorption compared to appropriate DOPAPCB-300/dopamine coated PDMS controls. Less than 10% increment was considered highly stable, between 10 – 30% increase was considered stable and 30% or greater was considered unstable.

**Statistical Analyses:** A single factor ANOVA (SPSS, Chicago IL) was run to determine statistical differences between controls (uncoated PDMS, and DOPAPCB-300/dopamine coated PDMS with no flow) and coated PDMS exposed to 1 dynes/cm², 6 dynes/cm² and 10 dynes/cm² shear stresses. A $p < 0.05$ was regarded as significant.

**Results and Discussion**

The exploded view of the flow cell design showing top and bottom mates, cell chamber, gasket channel circuit connectors are shown in Figure 3A and Figures 3B and 3C are the flow cell and recirculation circuit prototypes. There were also no observable leaks or air bubbles in the circuit during all runs. The 5 mL syringe in Figure 3C was used to
prime and extract trapped bubbles during priming.

As presented in Figure 4, the rpm to flow rate calibration of pumps showed linear relationships between the two variables although there were some pump-pump variation indicated by the rpm-to-pump data fitting equations. The coefficient of determination, \( R^2 \), for pumps 1, 2, 3 and 4 were 0.99, 1, 0.99, and 0.99 respectively.

![Graph showing the rpm to flow rate calibration of pumps with linear relationships and equations](Image)

**Figure 4.** Revolution per minute (rpm) to volumetric flow (mL/min) calibration curves of four perfusion pumps 1, 2, 3, and 4 that were used to recirculate phosphate buffered saline over DOPA-PCB-300/dopamine coated samples.

Each pump’s rpm-to-flow rate output provided guidance to obtain desired flow rates. Knowing the flow rates and obtaining the average flow velocity by dividing flow rates by the cross-sectional area of the cell chamber, sample or wall shear stress could be calculated using the \( \tau_o \) equation from the methods section. **Figure 5** shows the wall shear stress on the primary axis as a function of flow rate and Reynolds number on the secondary axis as a function of flow rate. After fitting calculated shear stress to flow rate...
date, it was determined that the calculated wall shear, $\tau_o$, increased with flow rate according to $\tau_o = 0.02 \left( \frac{\text{dynes} \times \text{min}}{\text{cm}^2 \times \text{mL}} \right) \times V \left( \frac{\text{mL}}{\text{min}} \right) - 4E - 15 \left( \frac{\text{dynes}}{\text{cm}^2} \right)$. In the shear stress calculation, the Darcy-Wiesbach friction factor depended on only the Reynolds number since the pre-calculated Reynolds number was $< 2300$. Shown also in Figure 5, we see that the Reynolds numbers calculated from the experimental flow rates and fluid properties were low and ranged from 0.25 – 1.25.

![Figure 5](image.png)

**Figure 5.** Physiologically relevant shear stresses (1 dynes/cm$^2$, 6 dynes/cm$^2$, and 10 dynes/cm$^2$) induced by laminar (Reynolds numbers 0.25 to 2) volumetric flows over samples placed inside flow cells.

Although the Reynolds numbers were low, a comparison of flow entrance length to cell chamber length was made to determine whether turbulent flow effects typical at flow entrances were dominant over entire length. The calculated entrance length, $L_e$, was 0.04 cm and compared to the cell chamber length = 6.35 cm, which is a two orders of magnitude bigger indicating that almost the entire sample surface and therefore the cell chambers saw fully developed laminar flows. Because the wall shear stress, $\tau_o$, remains constant along the flow direction in the fully developed regions of both turbulent...
or laminar flows, it was also deduced that almost all of the sample surface area saw
constant non-zero shear stresses during flow. However, in the entrance region, $\tau_o$ isn’t
constant but rather starts out larger before decreasing to a constant stress in the fully
developed region for any given flow rate. Therefore flow-induced erosion of DOPA-
PCB-300/dopamine may be possible and perhaps higher in the entrance length region
than what may occur in the fully developed flow region. It should be noted that the
scenario described above only reflects shear stress dynamics in a single fluid flow pass
while the continued interaction between the velocity profile and the samples from
multiple passes may further influence coating stability. Subsequent passes may cause
repeated interferences of the fully developed flow profile at the tubing/flow cell
connection and lead to repeated and transient increases in shear stress in the entrance
length region which may further influence the stability of the coating especially in high
shear stress test conditions. This theory is supported by the fibrinogen adsorption data
from coated samples that were exposed to shear stresses. Fibrinogen fouling before
flows on DOPA-PCB-300/dopamine coated PDMS (5.73 ± 1.97%) was significantly
lower than uncoated PDMS (100%, $p < 0.001$) as shown in Figure 6. The data shows
that fibrinogen fouling on coated samples increase with increasing shear stress
although to levels not significantly different from control (coated samples not loaded with
shear stress). In addition, fouling on coated PDMS with zero shear stress, although
lower, was not significantly different from coated samples that were exposed to 1
dynes/cm² (9.55% ± 0.09%, p = 0.23), 6 dynes/cm² (15.92% ± 10.88%, p = 0.14), and 10 dynes/cm² (21.62% ± 13.68%, p = 0.08). Our findings show that DOPApCB-300/dopamine coating were stable under the test conditions and only minimal coating erosion was observed. Compared to the coated PDMS no shear stress case, coated surfaces that were exposed to 1, 6, and 10 dynes/cm² of shear stress, adsorbed 3.83%, 10.20%, and 15.90% more fibrinogen respectively. It should be noted that the experiment was conducted at room temperature and with pH 7 PBS which are different from in-vivo conditions where the surface will interact with blood flow at a higher temperature. The study design used here however allows for a direct measurement of nonspecific protein adsorption on coated model surfaces after they have been exposed to flow, allowing for quantification of the stability of any non-fouling coating or surface immobilized enzymes against shear stress. Contamination of sample surfaces with biological material from stability testing with whole blood and perhaps plasma, on the
other hand, could lead to unreliable coating stability data using this approach. Non-specific protein fouling on coatings exposed to blood or plasma flow shear stress would be simply difficult to evaluate.

**Conclusion**

In this study, the stability of a low-fouling DOPA-PCB-300/dopamine coating against various flow-induced shear stresses was measured. It was found that instability, as measured by percent increase in fibrinogen fouling between shear treated and no shear samples, increases with shear stress. To conduct the experiment, flow cells were fabricated and characterized for flows that yield different shear stresses (1 dyne/cm², 6 dyne/cm², and 10 dyne/cm²). The surfaces of PDMS membranes were then coated with low fouling DOPA-PCB-300/dopamine followed by testing of the coating's stability against those shear stresses by placing the coated PDMS samples inside the flow cells and recirculating PBS over the samples at given flow rates for 8 hrs. Fibrinogen fouling between shear stress and no shear stress coated samples were compared to determine differences. Less than 10% increment was considered highly stable, between 10 – 30% increase was considered stable and 30% or greater was considered unstable.

Compared to the coated PDMS with no shear stress case, coated surfaces that were exposed to 1, 6, and 10 dyne/cm² of shear stress, respectively adsorbed 3.83%, 10.20%, and 15.90% more fibrinogen. Our results therefore show that DOPApCB-300/dopamine coating were stable and only minimal coating erosion was observed. As newer and more robust anticlotting coatings get developed, this simple and easy-to-use in-vitro flow cell system provides an appropriate pre-in vivo screening tool for determining coating stability under flow before conducting animal testing. The flow
system can be used to evaluate many coatings and surface modifications in biomaterials and blood-contacting devices. Other biomarkers for blood coagulation can be studied with this flow cell as well as evaluating the effects of pH and temperature.

The gas transfer properties of polymeric materials and interrogation of human cells and microorganisms such as bacteria and viruses with polymer permeable agents like nitric oxide could also be studied taking advantage of the dual chamber design of the flow cell. The results here suggest that coating erosion play a role in reducing the effectiveness of anti-fouling coatings used on blood-contacting medical devices.

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