Evaluation of Novel Hemocompatible Surface Coatings for Extracorporeal Life Support: A Biocompatible Alternative to Systemic Anticoagulation

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Evaluation of Novel Hemocompatible Surface Coatings for Extracorporeal Life Support: A Biocompatible Alternative to Systemic Anticoagulation

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Keywords: extracorporeal life support, biomaterials, coagulation, extracorporeal membrane oxygenation, metal organic framework, perfluorocarbons

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Dedication

For battlefield wounded and the heroes that fight to save them
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Abstract

Extracorporeal life support (ECLS) is a class of technologies used to support or replace the function of failing organs. During ECLS, blood is withdrawn from systemic circulation and circulated through an artificial organ or “treatment membrane” that performs the function of the failing organ, prior to return to systemic circulation. While ECLS provides life-saving therapy to wide patient populations from pre-term infants to combat-wounded soldiers, this therapy is limited due to secondary thrombotic and bleeding complications that result from: 1) exposure of blood to the foreign surfaces in the device circuit and 2) administration of anticoagulant drugs to prevent clot formation in the circuit.

In this study, we assessed a biomaterial solution to the challenge of hemostasis during ECLS by modifying surfaces in the circuit to improve compatibility with blood. This approach would provide local coagulation management at the blood-biomaterial interface, obviating the use of systemic anticoagulant drugs that cause secondary bleeding. We investigated a non-adhesive, liquid-infused coating called tethered liquid perfluorocarbon (TLP) that prevents plasma protein adsorption. We also investigated a metal-organic framework that catalyzes nitric oxide release from endogenous donors, localizing the platelet inhibitory effects of nitric oxide to the biomaterial surface as occurs in the endothelium. Our objective was to determine if these coatings were a robust biomaterials solution for ECLS without administration of anticoagulant drugs. We developed a three-step approach to assess the efficacy and safety of biomaterials for ECLS. First, materials were first evaluated in vitro in healthy donor blood using thromboelastography and
platelet aggregometry. Second, we proceeded with evaluation of TLP applied to complete ECLS circuits in vivo using a swine model for 6 hours of circulation. We assessed thrombus formation by scanning electron microscopy, coagulation function using clinical tests, gas exchange performance of the membrane using pre- and post-membrane blood gases and assessed safety using vital signs and histology. Finally we evaluated TLP in a 72-hour intensive care unit study without supplemental anticoagulation utilizing similar methods as described in our 6 hour model, with additional analysis of mechanical ventilation settings, systemic cytokine expression, hematology and protein adhesion. We hypothesized that TLP would enable 72 hours of heparin-free ECLS by inhibiting protein adsorption, preventing thrombotic circuit occlusion and preserving native blood parameters; all without impeding membrane performance or causing systemic complications.

Both TLP and the nitric oxide catalyst reduced the time and rate of thrombus initiation as well as clot strength ex vivo. The nitric oxide catalyst also reduced platelet aggregation. In our 6 hour evaluation, TLP applied to ECLS circuits reduced thrombus formation compared to control, heparin-coated circuits and did not affect gas transfer across the membrane lung or cause untoward effects. In our 72 hour evaluation, TLP failed to prevent thrombotic circuit occlusion, and additionally altered the performance of the membrane lung requiring greater support from the mechanical ventilator compared to control animals that received heparin-coated ECLS circuits with systemic anticoagulation therapy. We concluded that TLP is currently not an efficacious solution to permit ECLS for 72 hours without anticoagulant drugs. Future studies are needed that utilize the three-step assessment method we have developed here to evaluate multi-functional biomaterials with combined ability to prevent protein adsorption and inhibit platelet activation, such as occurs in the endothelium.
Chapter One

Extracorporeal life support: history, development and future applications

1.1 Abstract.

Extracorporeal life support (ECLS) was developed for temporary, partial replacement of cardiopulmonary function but has evolved to include technologies for extended support for multiple forms of organ failure. This therapy was originally highly invasive, required continuous support by expert clinicians, necessitated extensive rudimentary equipment and resources, and posed significant risk to the patient. Today this therapy has become a life-saving intervention for broad patient populations from neonates to adults, utilizing advanced modern, portable technology. Future plans for minimally invasive ECLS that can be used out of hospital for support in the patient’s home; and incorporation of ECLS technology into autonomous critical care platforms to be piloted to the site of battlefield injury are under development. Despite technological advances, one paramount challenge limits widespread use of ECLS – management of coagulation. Exposure of blood to foreign surfaces and sheer stress in the extracorporeal circuit invokes damage to blood cells and causes circuit thrombosis. Administration of anticoagulant drugs to prevent thrombosis causes secondary bleeding complications. This dissertation proposes a novel solution to this challenge of hemostasis by improving the biocompatibility of the materials used to construct the ECLS circuit; and outlines a methodological roadmap for evaluation of these biomaterials. This chapter introduces ECLS technology and describes the current state and future directions for this therapy.
1.2 Introduction.

ECLS is a class of technologies used to support or replace the function of failing organs – to include the lungs, liver, heart, and kidneys. The prefix “extra-”, meaning “outside”, and “-corporeal”, or “pertaining to the body”, describes the general function of these devices which remove blood from the systemic circulation and direct it outside the body to an external treatment membrane or artificial organ. The treatment membrane performs the function of the failing organ (e.g. add oxygen (O₂) to the blood and remove carbon dioxide (CO₂) for pulmonary support). The treated blood is then returned to the systemic circulation. This therapy was initially utilized in the operating room for cardiopulmonary support during invasive surgical procedures and was eventually modified for use in the intensive care unit (ICU) for extended duration of support (1). Today this technology is utilized outside the ICU in deployed military surgical hospitals (2-4) and in ground and aeromedical transport of unstable patients (5-7). Future applications such as extended support for patients awaiting an organ transplant (“bridge to transplant”) or destination therapies for those with chronic diseases, such as Chronic Obstructive Pulmonary Disease (COPD), are under development (8). These applications will depend on improving the safety profile of the devices and developing miniaturized, wearable technologies that can be operated out of the hospital in the patient’s home. The major hurdle limiting development of such devices is selection of biocompatible materials that reduce thrombotic, hemorrhagic and inflammatory complications associated with blood-foreign surface interactions in the ECLS circuit.

1.3 The ECLS circuit: an overview.

The ECLS circuit consists of the following five key components: 1) treatment membrane/artificial organ, 2) blood pump, 3) vascular access catheter(s), 4) connective tubing, 5) heat exchanger (Figure 1.1). Each of these components can be modified depending on the
Figure 1.1 A) Image of extracorporeal life support device for partial lung support (Maquet Getinge Group, Rastatt, Germany). Picture includes the key components of an extracorporeal circuit: 1) treatment membrane, 2) blood pump, 3) vascular access catheter(s), 4) connective tubing, 5) heat exchanger. B) The treatment membrane shown is composed of hollow gas-exchange fibers (see schematic of oxygen ($\text{O}_2$) diffusion out of the fiber lumen into the blood, and carbon dioxide ($\text{CO}_2$) diffusion from the blood into the fiber lumen. C) The treatment membrane also includes an integrated heat exchanger depicted with heated water ($\text{H}_2\text{O}$) flowing through the lumen of the heat exchange fibers, transferring heat to blood passing around the fibers.
indication for use and the physiological requirements of the patient. For the purpose of this review, we will focus on ECLS technologies for pulmonary support, as these devices will be the focus of the research described in this dissertation.

1.3.1 Treatment membrane. The treatment membrane is the most variable component of the ECLS circuit, as it determines the type of support delivered (renal, hepatic, pulmonary, cardiac, etc.) and can be modified based on the size (e.g. neonates versus adults) and physiological demands (partial or total organ support) of the patient. A treatment membrane designed for pulmonary support, also referred to as a “membrane lung”, is designed to add O\(_2\) to venous blood and remove CO\(_2\). These membranes consist of a series of hollow fibers encased in a plastic housing. Gas (called the “sweep gas” -- usually 100% O\(_2\)) is ventilated through the lumen of the hollow gas-exchange fibers. Venous blood enters the membrane and contacts the outside of the gas exchange fibers where gas transfer – addition of O\(_2\) from the sweep gas into the blood and removal of CO\(_2\) from the blood into the sweep gas – occurs by diffusion (Figure 1.1, Panel B). The blood exiting the membrane is oxygenated, with CO\(_2\) reduced, prior to being returned to the patient’s systemic circulation. The size of the membrane is modified based on the metabolic demands of the patient. For example, a membrane with a greater surface area for gas diffusion and larger blood path to maximize gas mixing and the rate of blood flow through the membrane will provide the highest level of support (1). The maximum rate of gas exchange (both O\(_2\) and CO\(_2\)) by the average membrane lung (200-400 mL/min) is comparable to and can exceed the gas exchange rate of the native lung at rest (200-250 mL/min) (9). This exemplifies the potent gas exchange capabilities of modern ECLS systems.

Gas exchange in the membrane lung is comparable to the human lung; and is subject to
similar limitations, albeit by different mechanisms. In the native lung, gas exchange depends on both proper ventilation, which is delivery of air into the alveolar space; and proper perfusion, which is delivery of blood to the pulmonary capillaries that encapsulate the alveoli (10). Gas exchange occurs across the alveolar-pulmonary capillary membrane where CO$_2$ diffuses from the mixed venous blood into the alveolar space, and O$_2$ diffuses from the alveolar space into the blood. In the membrane lung, the sweep gas is ventilated through the air space/inner lumen of the gas exchange fibers; and perfusion occurs as blood is pumped into the membrane and surrounds the gas exchange fibers. If an area of the lung is perfused but the air space is obstructed, gas exchange cannot occur causing pulmonary shunt (11). For example, if the alveoli are filled with edematous fluid or become consolidated during pneumonia, O$_2$ will not be available to the blood passing through the associated capillaries, and the gradient for CO$_2$ removal will be absent. In the membrane lung, if the lumen of the gas exchange fibers in the membrane becomes filled with fluid, for example due to sweep gas condensate or plasma leakage through the fibers into the lumen, this is reminiscent to pulmonary shunt, limiting gas exchange. If a pulmonary embolism or occlusive thrombus prevents blood flow to pulmonary capillaries, the O$_2$ in the associated alveoli would not be accessible and no CO$_2$ could be offloaded, causing pulmonary dead space (11). Similarly, if an occlusive thrombus prevents blood flow through a region of the membrane lung, the sweep gas in those fibers cannot be accessed for gas exchange, causing a form of dead space (9).

In the blood, O$_2$ and CO$_2$ are transported to and from the tissue by different mechanisms. Because of this, the rate of transfer of O$_2$ and CO$_2$ by the native lung and membrane lung are controlled by different methods. The total O$_2$ content of the blood is the sum of the dissolved O$_2$ and hemoglobin-bound O$_2$. The solubility of O$_2$ in blood is low (0.0031 mL / mmHg O$_2$ / dL blood) such that the dissolved O$_2$ in blood generally represents < 1% of the total O$_2$ content in clinical
practice (12, 13). This means that the majority of O\textsubscript{2} in the blood is bound to hemoglobin, and only small increases in O\textsubscript{2} content can be achieved once hemoglobin is saturated (13). The O\textsubscript{2} content of the blood and rate of delivery of blood to the tissue – the cardiac output (heart rate \times stroke volume) – determine oxygen delivery to the tissue (10). Likewise, the major factors that determine oxygenation by the membrane lung are the O\textsubscript{2} carrying capacity of the blood (dependent on hemoglobin concentration, CO\textsubscript{2} content and oxyhemoglobin saturation of the blood), and the rate of blood flow through the circuit (12, 14). When the metabolic needs of the patient cannot be supported by the membrane lung, the blood flow rate through the membrane must be increased (similar to an increase in cardiac output in the body) or hemoglobin concentration must be increased by transfusion (12). In this way, O\textsubscript{2} transfer by the ECLS circuit is generally perfusion limited.

CO\textsubscript{2} is \textasciitilde20 times more soluble in blood and is also more diffusible than O\textsubscript{2}, such that the CO\textsubscript{2} elimination capacity of the membrane is almost always greater than the oxygenation capacity (12, 15). This means that for patients requiring CO\textsubscript{2} removal without oxygenation support, lower blood flow rates and smaller membrane lungs can be utilized (12). CO\textsubscript{2} is carried in the blood in three main forms: 1) dissolved (~5-10\%), 2) reversibly bound to the amino terminal of blood borne proteins forming carbamino compounds (~5-30\%) or 3) hydrolyzed to form the bicarbonate ion (~60-90\%) (15). In this way, the major factors that determine CO\textsubscript{2} removal by the membrane lung are the partial pressure gradient of CO\textsubscript{2} across the membrane and the sweep gas delivery rate. The sweep gas flow is generally adjusted to achieve a target systemic P\textsubscript{CO2} (12). Unlike oxygenation, CO\textsubscript{2} removal is more dependent on sweep gas flow rate (ventilation) than the blood flow rate (1, 9, 12). Albeit, CO\textsubscript{2} removal is still positively correlated with blood flow as demonstrated by Park and colleagues (14).
1.3.2 Blood pump. The blood pump drives the flow of blood from the patient, through the ECLS tubing and membrane, and back to the patient’s systemic circulation. The two primary types of pumps utilized for ECLS are peristaltic pumps and centrifugal pumps. Extracorporeal Life Support Organization (ELSO) registry report indicates a trend in increased centrifugal pump usage versus peristaltic pumps over the past 15 years, with the exception of pediatric populations (1). The pumps are designed to support high (2-10 L / min) or low (<2 L / min) blood flow rates depending on the indication of use (1). The most common pump-related complication is hemolysis, largely due to shear stress, but also can include tubing cavitation and excessive negative pressure, high outlet pressure, power failure and thrombosis (1). While most pumps provide continuous flow, certain applications for pulsatile blood flow have been developed.

1.3.3 Vascular access catheter(s). The ECLS catheter is the point of access by which blood is withdrawn and/or returned to the systemic circulation. The size of the catheter varies based on blood flow rate requirements, where larger catheters are needed to support higher flow rates. There are two main methods of cannulation for ECLS: 1) veno-arterial (VA) ECLS – where blood is withdrawn from a major vein and returned to an artery; and 2) veno-venous (VV) ECLS – where blood is withdrawn and reinfused into venous circulation (1). For pulmonary ECLS, VA cannulation provides pulmonary and circulatory support, while VV ECLS provides primarily pulmonary support; although, some data suggests that VV ECLS may unload the function of the right heart providing cardiac benefits (16). VV ECLS also indirectly provides circulatory support by allowing for reduction in mechanical ventilator settings, decreasing the negative effects of mechanical ventilation on systemic blood pressure (17). For VV ECLS, catheters can be placed into two separate veins (dual-site cannulation) or in one vein using a dual-lumen catheter. During VV ECLS with single-site cannulation a dual-lumen catheter is placed into the right jugular vein
Venous blood is withdrawn from the superior and inferior vena cava into the outer lumen of the catheter, where it is then circulated through the ECLS circuit. Blood from the ECLS circuit is then reinfused into the right atrium, where it is pumped through the pulmonary circulation and out to the systemic circulation (1). Single-site cannulation is becoming the standard method for pulmonary applications and is the method of cannulation utilized in this dissertation.

1.3.4 Connective tubing. The connective tubing links all components of the ECLS circuit. The tubing is generally composed of polyvinylchloride (PVC) combined with a plasticizer to increase the flexibility of the tubing. A minimal tubing length is desired to decrease resistance to blood flow in the circuit; however, the length of the tubing is also an important consideration for mobilization of the patient and prevention of circuit disconnects (1). The inner diameter of the tubing is selected based on the desired blood flow rate, where a larger internal diameter supports a higher rate of flow at a constant pressure (18).

1.3.5 Heat exchanger. During extracorporeal circulation, blood is removed from the body and exposed to ambient temperature, cooling the patient. To maintain normothermia, a heat exchanger is often incorporated into the treatment membrane or direct blood warming can be applied. When the heater is incorporated into the treatment membrane, heated water is circulated within an additional set of fibers (separate from the hollow gas exchange fibers) and heat is transferred (rather than gas) as blood passes over the surface (Figure 1.1, Panel C) (1).

1.4 A brief history of ECLS.

The first successful clinical usage of ECLS was performed in 1953 by Dr. John H. Gibbon Jr. (1). This initial device, which weighed over 1,000 kg and required two technicians to operate,
provided 26 minutes of cardiopulmonary support to a young woman with right ventricular failure during repair of an atrial septal defect (19). The invention of silicone rubber for durable, gas-permeable silicone membrane lungs; as well as advances in controlled management of anticoagulation administration allowed for extended use of ECLS, for several hours, in the operating room and the post-operative recovery period (1). In 1973, the first reported use of ECLS outside the operating room in the intensive care unit was performed by Dr. J.D. Hill for 75 hours of cardiopulmonary support in a trauma patient with a ruptured aorta and traumatic acute respiratory distress syndrome (ARDS) (20). The patient survived to discharge. In 1985, Dr. Robert Bartlett, the “father of extracorporeal membrane oxygenation (ECMO)” in the USA, pioneered the use of ECLS in neonatal populations for respiratory diseases (21). His success brought attention to ECLS technology and led to an increase in ECLS centers from 18 to 100 word-wide in the 1990s (20).

Adoption of ECLS in adult populations was much slower than in pediatric and neonatal populations, and a series of randomized controlled trials in the 1990s and early 2000s failed to demonstrate a clear survival benefit. These trials were complicated by a lack of ECLS expertise in many centers, use of dated equipment that was highly invasive and caused significant blood trauma, and selection of declining patients in whom ECLS would be a last resort therapy (1, 22, 23). At that time, use of ECLS was still highly invasive, requiring complete sedation and intubation of the patient, continuous support and supervision from a specialist, and was accompanied with major bleeding complications (8). In 2009, advancements in ECLS technology and an increases in ECLS center expertise were realized and demonstrated to the medical community in the “Conventional Ventilatory Support Versus Extracorporeal Membrane Oxygenation for Severe Adult Respiratory Failure” (CESAR) randomized control trial, where patients assigned to ECLS
therapy had a 63% survival rate as opposed to 47% in the conventional management group, showing the significant survival benefit from ECLS in adults with reversible respiratory failure (24). The results of this trial were released prior to the 2009 H1N1 influenza pandemic when a rapid rise in cases of severe adult respiratory failure presented a clear need for ECLS when mechanical ventilation was insufficient to support the respiratory demands of the patient or exacerbated lung injury (25, 26). This led to an increase in the number of worldwide ECLS centers to 467 by 2016 (1).

1.5 ECLS today.

At the same time as the CESAR trial and H1N1 pandemic, ECLS technology began to evolve such that the therapy could be carried out in a significantly less invasive manner. Treatment membranes were miniaturized due to optimization of blood flow dynamics and utilization of highly gas permeable materials that were durable and resistant to plasma leakage, allowing for sufficient gas exchange using a smaller surface area for gas transfer than previously required (27). Blood pumps were miniaturized and configured to reduce hemolysis. Miniaturization of ECLS circuits allowed for use of smaller vascular access catheters; and surgical practice advanced to allow for percutaneous catheter placement, rather than invasive surgical operation (1). Biocompatible coatings were developed to prevent blood disturbance by the foreign materials in the circuit, which will be discussed in detail in the next chapters (28). It is important to note that to date, the coated materials still require administration of systemic anticoagulant drugs to prevent thrombosis. These advances in ECLS technology now allowed for patients receiving therapy to be awake, spontaneously breathing, extubated and ambulatory with assistance (8).

These technological advances reduced invasiveness of ECLS therapy and led to a marked increase in utilization. As of July 2018, there have been over 100,000 ECLS cases across
disciplines reported in the Extracorporeal Life Support Organization Registry (29). Specifically for adult respiratory support, there has been an increase from 200 cases in 2008 (prior to release of the CESAR trial and H1N1 pandemic) to 2,732 cases in 2017. The average duration of support increased from 195 hours in 2008 to 271 hours in 2017 (29).

In addition to increased utilization in the general population an important modern application for ECLS, which is the primary motivation behind the research described in this dissertation, is to support combat casualties and battlefield wounded. The United States military currently utilizes ECLS outside the ICU in deployed military hospitals and on rotary and fixed wing aircrafts to support combat casualties with trauma-induced organ failure (30, 31). ECLS for both cardiopulmonary (2) and renal support (3, 4) has been successfully utilized in military hospitals, and has proven to be safe and effective during local and transcontinental aeromedical patient transport (32, 33). This capability was only possible following miniaturization of ECLS technology and improvements in ease of operation as described above. To date, ECLS for combat casualties cannot be performed at the point of injury, and requires initial stabilization and transport of the patient to a combat hospital with specialized surgical capabilities (34).

Despite the technological advances mentioned above that have expanded utilization of ECLS, complications still remain. Many centers report hemorrhagic complications from blood trauma and anticoagulant administration as the most common complication for ECLS patients (estimated 30-60%, but as high as 100% in some cohorts); however the reported rate is highly variable due to inconsistent methods for determination of “bleeding episodes” (35-38). Bleeding and blood stress require most patients to receive blood transfusions, which is associated with increased morbidity and mortality (39). While anticoagulant administration to prevent thrombosis results in frequent bleeding, ELSO reports that from 2013 to the present the most frequently
reported mechanical complication during ECLS for adult respiratory care is circuit clotting, seen in 16.4% of cases (29). The second most common mechanical complication was membrane lung failure (6.1%). Additionally, infectious complications were reported in 14.5% of cases and reduced the rate of survival to 52% (29). Solutions to these complications will be the subject of this dissertation.

1.6 ECLS future applications.

Utilization of ECLS is expected to continue to grow, with hopes for extracorporeal respiratory support to become as common as mechanical ventilation in the ICU (8). Increased usage and provider familiarity would allow for conventional patient management, without continuous oversight of specialists. Improvements in biocompatibility of ECLS materials and optimization of flow patterns to eliminate hemolysis could reduce the need for systemic anticoagulant drugs (40). This would address the high instance of bleeding complications currently observed and allow for patients to be supported for months at a time, rather than the average 1-2 weeks of support that is current practice (1, 8). Development of miniaturized wearable ECLS devices would allow for long-term bridge-to-transplant patients and chronic support patients to receive therapy out of hospital in their own homes. New applications, such as support of pre-term neonates using an “artificial placenta” that consists of ECLS circulation via the jugular and umbilical vein in lieu of mechanical ventilation while lungs are filled with fluid, have shown promise in laboratory settings (41). Multi-functional membranes that combine different organ support functions within one treatment membrane could provide support for multi-organ failure without increasing blood-contact area, flow-related stress or logistical impact.

U.S. military physicians are interested in adapting ECLS technology to allow for use of the devices on the battlefield at the point of injury (34). This would be a life-saving intervention by
providing cardiopulmonary stabilization prior to patient transport and during damage-control surgical interventions. Additionally, because ECLS allows for control of cardiac output, oxygenation, metabolite removal, temperature and more this technology could be incorporated into self-regulated, autonomous critical care platforms. These platforms containing ECLS technologies could be remotely piloted to the site of injury where a medic could initiate extracorporeal circulation. The casualty could then be evacuated from the battlefield while being remotely monitored by a clinician during transport to a higher echelon of care. Still, in discussion of the feasibility of ECLS on the battlefield, the need to administer anticoagulants is identified as a major challenge, especially in treatment of patients with coagulopathies induced by trauma and the austere field care environment (34).

1.7 Conclusions.

ECLS technology has evolved significantly since its introduction in the 1970s. Advances in safety and feasibility of use have sparked rapid increase in utilization and inspired physician scientists to develop novel applications for ECLS. Still, development and adoption of these novel therapies require the challenge of hemostasis during ECLS to be resolved. Despite advances in cannulation procedures, reduction in membrane size and blood contact area, increased blood-material compatibility and reductions in pump-related hemolysis, bleeding and thrombotic complications are still exceedingly high and caution practitioners to utilize this life-saving therapy. Future applications for home-wear devices, extended patient support and early intervention for traumatic injury are dependent on a solution to ECLS-induced coagulopathy. Understanding the origins of ECLS, developments in the past decades and scope of proposed future applications demonstrates a clear need for secondary bleeding and thrombotic complications to be resolved. Novel solutions to this challenge will be the subject of this dissertation.
1.8 References.


10. West JB. Respiratory physiology: the essentials: Lippincott Williams & Wilkins; 2012.


Chapter Two:

Extracorporeal life support and the incessant challenge of coagulation

2.1 Introduction.

Management of coagulation during extracorporeal life support (ECLS) is a significant challenge where both thrombotic and bleeding complications occur, often simultaneously. The vascular endothelium actively maintains blood fluidity and hemostasis via a delicate balance of pro- and anti-thrombotic factors. When blood is pumped outside the endothelium into the ECLS circuit foreign surface contact, shear stress and variable flow activate coagulation and cause inflammation. This results in thrombus formation, which can occur in the ECLS membrane, pump, tubing and catheters. Return of activated blood to the patient’s circulation can cause systemic thrombosis, and circuit clots can become dislodged and cause life-threatening thromboembolism. Systemic anticoagulant drugs are administered to prevent thrombosis; however, these anticoagulants can be difficult to titrate, especially in patients with underlying coagulopathy. This can lead to life-threatening bleeding complications. To understand the complex challenge of coagulation management during ECLS, we first describe coagulation and maintenance of blood fluidity as it occurs in the endothelium – highlighting key pro- and anti-inflammatory regulators. We then describe key alterations to hemostasis during ECLS, discussing how blood chemistry, biomaterial surface characteristics and blood flow determine the coagulation response. Finally, we describe how coagulation is managed clinically during ECLS, prior to discussion of alternative management strategies.
2.2 Hemostasis in the vascular endothelium.

The endothelium serves as a blood-compatible vascular lining in vertebrates and is estimated to cover a surface area of 3,000 to 6,000 m² in an adult human (1). Once thought to be a passive barrier separating blood from foreign sources of activation, the vascular endothelium is now understood to be a highly active surface that not only regulates expression of pro- and anti-thrombotic factors to preserve blood fluidity and prevent thrombus formation and bleeding; but also provides targeted delivery of immune cells, regulation of vascular tone and perfusion, and support of healing through angiogenesis following inflammatory injury or tissue trauma – in addition to countless location and tissue-specific functions (1, 2). The endothelium regulates coagulation through expression of surface receptors, alteration of flow distribution, control of fluid transfer and permeability, as well as secretion of regulatory molecules and factors that mediate blood cell activity (3). Here we describe a general overview of the coagulation and fibrinolytic systems, and then discuss key endothelial surface receptors and secreted factors that regulate hemostasis -- understanding that many of these mediators play a joint role in the tightly integrated systems of coagulation and inflammation.

2.2.1 Overview of coagulation. The balance of thrombosis and bleeding is controlled by interactions between the coagulation, fibrinolytic and inflammatory systems, as well as the endothelium, blood cells and platelets. Coagulation was initially described as a “cascade” model where a series of enzymatic reactions were initiated along one of two distinct pathways (the extrinsic and intrinsic pathways) that converged at a common pathway (Factor Xa) which led to thrombin generation and fibrin activation (4). This model describes coagulation as it occurs in plasma, but does not account for the interaction of endothelial, blood and inflammatory cell surfaces (4). The more recent “cell-based” model of coagulation emphasizes the active role of
cellular surfaces in regulating coagulation activity (Figure 2.1) (5). The cell-based model is divided into 3 phases: 1) initiation, 2) amplification and 3) propagation (6). The initiation phase occurs on tissue factor (TF) bearing surfaces – such as fibroblasts, damaged/stimulated endothelium, activated monocytes, and cellular fragments/microparticles (7). Circulating plasma Factor VII binds surface-bound TF and becomes activated, leading to subsequent activation of Factor X and a small amount of thrombin formation (6). The amplification phase occurs as platelets adhere to subendothelial matrix proteins at the site of vascular injury, which causes the platelets to become partially activated and localizes them to the site of TF release. Then, thrombin released in the initiation phase fully activates the platelets, as well as other co-factors (Factors V, VIII and XI), to prepare the platelet for pro-thrombinase complex formation and thrombin burst (4, 6). Activated platelets tightly bind circulating Factors IXa, Xa, and XI, and thrombin mediates adhesion and activation of von Willebrand Factor (vWF) bound-Factor VIII (6). In the propagation phase, the tenase complex (Factor VIIIa/IXa) assembles on the platelet surface, causing Factor X activation and prothrombinase complex (Factor Va/Xa) formation. The assembly of the prothrombinase complex leads to rapid thrombin (Factor IIa) formation, or “thrombin burst.” Thrombin cleaves fibrinogen to fibrin, which polymerizes to form a stable fibrin clot from the initial platelet plug (6). This model demonstrates how platelets serve as the surface on which the majority of thrombin required for clot formation occurs (6).

2.2.2 Overview of fibrinolysis. The fibrinolytic system counteracts thrombosis by dissolving the fibrin clot into soluble degradation products. This process occurs through conversion of plasminogen, a circulating zymogen, to the active form plasmin. Plasminogen associates with fibrinogen in the circulation so that when fibrin forms and cross-links, plasminogen is incorporated within the clot and is in close proximity to fibrin prior to activation to plasmin (8).
**Figure 2.1** The proposed cell-based model of coagulation consisting of the following three key phases: initiation, amplification and propagation. Reproduced with permission of Bentham Plasminogen Science Publishers Ltd. from Frederick R, Pochet L, Charlier B, Masereel B. Modulators of the coagulation cascade: focus and recent advances in inhibitors of tissue factor, factor VIIa and their complex. *Current Medicinal Chemistry.* 2005;12(4): 397-417.
There are multiple plasminogen activators, such as tissue-type plasminogen activator (t-PA), which is constitutively expressed by the endothelium, as well as urinary-type plasminogen activator (u-PA), high molecular weight kininogen, prekallikrein and Factor XII (8). t-PA and u-PA are inhibited primarily by plasminogen activator inhibitor-1 (PAI-1), which is synthesized by endothelial cells, platelets and other cell types to reduce fibrinolysis (8, 9).

2.2.3 Pro-coagulant factors in the endothelium. Pro-coagulant, anti-fibrinolytic factors are generally not constitutively expressed on the endothelium but are primarily expressed or released in response to vascular injury, inflammation and other pathological responses.

2.2.3.1 Tissue factor (TF). TF is a transmembrane protein that is not normally present on circulating blood or endothelial cells but is constitutively expressed on cells surrounding the endothelium such as smooth muscle cells and fibroblasts (10). When the endothelial barrier is compromised, TF from the surrounding cells comes into contact with blood and initiates thrombosis through formation of the TF/Factor VIIa complex (11). Cytokines and inflammatory complexes can also stimulate TF expression in monocytes and endothelial cells, promoting pathological, immune-mediated thrombogenesis (10).

2.2.3.2 von Willebrand factor (vWF). vWF is synthesized in endothelial cells and megakaryocytes and stored for secretion in Weibel-Palade bodies and platelet α-granules (3). When the endothelial surface is compromised, vasoactive substances such as thrombin, histamine or bradykinin trigger vWF release to the endothelial surface where it contributes to platelet adhesion to the sub-endothelial matrix via Gp1bα; or in instances of high flow promotes platelet aggregation by binding glycoprotein IIb/IIIa and stabilizing interactions between platelets and collagen (3, 12). vWF also carries and stabilizes Factor VIII, which is a part of the tenase complex that activates Factor Xa leading to conversion of
prothrombin to thrombin (13). vWF has been well-utilized as a plasma marker of endothelial damage (14).

2.2.3.3 Platelet activating factor (PAF). PAF is synthesized in endothelial cells in response to agonists such as thrombin and bradykinin. PAF promotes recruitment and activation of leukocytes at the endothelial surface where leukocytes release pro-coagulant factors, provide binding sites for fibrinogen and associate with platelets to cause further platelet and leukocyte activation (15, 16).

2.2.3.4 Plasminogen activator inhibitor 1 (PAI-1). PAI-1 is the most ubiquitously found plasminogen activator inhibitor and is the primary inhibitor of t-PA and u-PA, decreasing fibrinolysis (17). It is released from endothelial cells when stimulated by certain pro-inflammatory cytokines, growth factors and lipoproteins. PAI-1 is also released by platelets, monocytes, macrophages and other cells (17).

2.2.4 Anti-coagulant factors in the endothelium. Anti-coagulant, pro-fibrinolytic factors are generally constitutively expressed on the endothelium to maintain blood fluidity and prevent thrombosis in the absence of injury or disease.

2.2.4.1 Tissue factor pathway inhibitor (TFPI). TFPI is a serine protease inhibitor that prevents thrombin generation by inhibiting factor Xa and the TF/Factor VIIa complex (17). TFPI is primarily synthesized in endothelial cells and remains on the endothelial surface bound to heparin sulfates or circulates in plasma bound to lipoproteins (18).

2.2.4.2 Tissue plasminogen activator (t-PA). t-PA activates the fibrinolytic system by converting plasminogen to plasmin. Its activity is increased as much as 500-fold in the presence of fibrin (19). t-PA is synthesized by endothelial cells from multiple sources in culture; however, known synthesis in vivo is primarily restricted to small vessels and
vascular branches (17). Synthesis and secretion of t-PA is stimulated by thrombin, bradykinin, oxygen free-radicals, laminar shear stress and other agents (20, 21).

2.2.4.3 Heparin-containing proteoglycans. Endothelial cells synthesize and secrete heparin and heparin sulfate, which is anchored to the endothelial surface by a proteoglycan core (22). Heparin sulfate binds to and accelerates the activity of Antithrombin III (ATIII), an enzyme that inhibits thrombin and Factor Xa; heparin binding also localizes ATIII on the endothelial surface (23). These proteoglycans are also key constituents of the endothelial glycocalyx, a membrane-bound surface layer of proteoglycans, glycoproteins and other soluble proteins that regulates endothelial permeability, responds to mechanical signals and contributes to the pathological response of the endothelium in a number of disease states (24).

2.2.4.4 Thrombomodulin (TM). Thrombomodulin is a protein receptor found on the endothelial surface that binds and inhibits thrombin. TM is primarily synthesized by endothelial cells and is upregulated in areas of high flow (arteries) and down-regulated in a pro-inflammatory environment (3). In addition to inhibiting thrombin-mediated fibrinogen activation, thrombin bound to TM activates the anti-coagulant protein C pathway (25).

2.2.4.5 Protein C and protein S. Protein C circulates in plasma in inactive form until it is cleaved by thrombin bound to TM, forming activated Protein C (aPC). aPC proteolytically degrades Factors Va and VIIIa and can increase fibrinolysis by neutralizing PAI-1 (26). Endothelial cells express the endothelial cell protein C receptor (EPCR) which binds protein C and accelerates its activation to aPC by thrombin-TM by ~20-fold (26). Protein S, which is also synthesized by the endothelium, is an important Protein C co-factor that
accelerates aPC activity and is thought to be instrumental in the close association of aPC with the phospholipid surface, which is necessary for its function (17, 27, 28).

2.2.4.6 Prostacyclin (PGI₂). PGI₂ is an eicosanoid constitutively secreted by the endothelium that inhibits platelet activity, promotes vasodilation and stimulates cytokine production (17). Release of PGI₂ is rapidly and temporarily elevated in response to thrombin, bradykinin, histamine and several cytokines (29).

2.2.4.7 Nitric oxide (NO). In the endothelium, NO is catalyzed by endothelial nitric oxide synthase (eNOS) which converts L-arginine to L-citrulline. The activity of eNOS is modified by several factors including shear stress, proinflammatory cytokine expression and ADP release (29). NO promotes vasodilation, reduces leukocyte adhesion to and migration through the endothelium, prevents vWF release from thrombin-stimulated Weibel-Palade bodies and inhibits platelet adhesion and activation (29). The potent anticoagulant effect of NO on platelet function, which will be later emphasized in this dissertation, has been well characterized (30, 31). NO reduces platelet activation and aggregation through cyclic guanosine monophosphate (cGMP) mediated mechanisms. Platelet agonists, such as collagen and ADP, activate signaling pathways that increase platelet intracellular Ca²⁺ concentrations ([Ca²⁺]ᵢ). Elevation of [Ca²⁺]ᵢ stimulates reorganization of the platelet cytoskeleton to form an activated conformation, induces expression of platelet surface activation makers and binding sites for aggregation, and triggers release of platelet granules that stimulate adjacent platelet activation (32). Endothelium-derived NO stimulates soluble guanylate cyclase, which increases platelet cGMP. cGMP mediates sequestration of Ca²⁺, reducing the [Ca²⁺]ᵢ and suppressing Ca²⁺ mediated platelet activation (33). In addition to endothelium-derived NO, platelets express
nitric oxide synthase and produce NO as a method of auto-inhibition, further demonstrating the importance of NO as a mediator of platelet function (34).

2.3 Altered hemostasis during ECLS.

The biomaterials that construct the ECLS circuit do not actively regulate homeostasis as occurs in the endothelium. Without a protective surface layer, such as the endothelial glycocalyx, or constitutive secretion of antithrombotic factors like NO and heparin, blood becomes activated upon exposure to the foreign surface. There are multiple factors that determine the compatibility of blood with the surfaces in the extracorporeal circuit. Virchow’s triad describes the three major factors that affect blood coagulation which are: 1) chemistry of the blood, 2) characteristics of the blood-contacting surface and 3) the blood flow path and rate (35). The impact of these key factors on coagulation during ECLS will be discussed in the next paragraphs.

2.3.1. Blood chemistry. The quantity, composition and activity of cellular elements (red and white blood cells, platelets), clotting factors and hemostatic regulators in the blood affect coagulation on the biomaterial surface (36). This can vary depending on species (37), gender (38), age (39, 40), genetics (41), injury status (42), temperature (43), pH and more (44). This is an important consideration for a diverse therapy like ECLS that is utilized for numerous pathological conditions. For example, blood from an elderly female with pneumonia receiving ECLS therapy for partial lung support in the intensive care unit will behave very differently than blood from a young combat casualty at an austere military hospital with pre-existing hemorrhagic shock receiving resuscitation and ECLS for cardiopulmonary support. This is also an important consideration for selection of blood for laboratory testing of biomaterials (36). In addition to the patient’s underlying blood chemistry, transfusion of blood products such as packed red blood cells (PRBCs) will alter blood composition and biomaterial interactions. Blood chemistry is the element
of Virchow’s triad that is currently targeted to prevent coagulation during ECLS through administration of anticoagulant drugs that affect the activity of key homeostatic regulators such as thrombin (45). This will be discussed in detail in section 2.5.

2.3.2 Biomaterial surface characteristics. The first reaction that occurs when blood is exposed to a foreign surface is rapid adsorption of plasma proteins (36). These adsorbed proteins then induce thrombosis by providing binding sites to localize platelets at the material surface and activating the platelets to a pro-coagulant state (46). The specific characteristics of the material surface affect the type and quantity of proteins that are adsorbed and the exposure of functional groups on the proteins that mediate thrombosis (46). Albumin, which accounts for over 50% of total plasma protein content (35-55 g/L), does not readily mediate platelet adhesion or activation and is thought to be relatively inert when adsorbed; whereas fibrinogen, which accounts for less than 10% of total plasma protein content (1.5-4.5 g/L), is considered the central mediator of platelet activation and aggregation on the biomaterial surface (47-49). Other plasma proteins such as immunoglobulins, vWF, fibronectin and vitronectin also induce platelet activation and/or adhesion to foreign surfaces (29). Sivaraman, and colleagues demonstrated that the degree of deformation of adsorbed fibrinogen, rather than total quantity of fibrinogen adsorbed is more strongly correlated with platelet activation and adhesion to the biomaterial surface (50). This demonstrates the importance of 1) the type of protein adsorbed (pro-coagulant vs inert) and 2) the interaction of the protein with the biomaterial surface to expose platelet binding sites in determining thrombogenicity of the material. In addition to plasma protein adsorption and subsequent platelet activation, biomaterial contact can activate plasma coagulation directly through activation of Factor XII (51, 52). Complement proteins such as C3 can also adsorb and activate, promoting coagulation and initiating inflammation (53, 54).
The characteristics of the material surface that affect the type and degree of protein adsorption and coagulation include the surface free energy, wettability, chemistry, topography and roughness (46). Surface free energy contributes to the interfacial energy between the material and the blood, determining if thermodynamics favor adsorption. It has been reported that protein adsorption is greater on surfaces with lower free energy (55). The wettability of the surface, described as the hydrophilicity, affects the strength of the interaction between the protein and the surface. Hydrophobic surfaces generally increase protein adhesion, favoring stronger interaction between the protein and the surface; whereas, hydrophilic surfaces form a layer of water that acts as a buffer to inhibit protein adhesion and reduce the strength of the protein-surface interaction (46, 56). Addition of functional groups and topographical features to the material surface can alter protein adsorption regardless of wettability. For example, surface texturing can increase surface hydrophobicity by trapping air between the topographical features, which inhibits protein contact with the surface forming a blood-repellant “superhydrophobic” material (57). The effect of surface roughness on protein adsorption is dependent on the size of the topographical features on the material, where nanoscale features may suppress adsorption, but microscale features may increase adsorption (58).

2.3.3 Blood flow, shear stress and hemolysis. The effects of blood flow, including rate and distribution, determine both the rate of transport of blood elements to the biomaterials surface and the level of shear stress at the surface. High flow rates cause “reaction controlled” surface adsorption, where blood cells and proteins are readily delivered to the surface and adsorption is highly dependent on surface characteristics (35). In low-flow systems, adsorption is “diffusion controlled,” dependent on availability of proteins and cells at the material surface (35). Shear stress can be understood by Poiseuille’s Law, which describes laminar flow of fluid in a cylindrical
container, such as blood in a segment of vasculature or connective tubing in the ECLS circuit (59). The velocity of the fluid is greatest in the center of the lumen and gradually decreases with proximity to the wall. Shear stress is the force that the near-wall flow exerts on the wall. This force is directly related to flow rate and fluid viscosity; and is inversely related to the vessel diameter (59). Therefore, it is important to select ECLS catheters and tubing that will support the flow rate needed for a specific type of ECLS therapy. Small catheters are easier to place but may cause excessive hemolysis if the diameter of the catheter lumen is insufficient to support the required flow. Shear stress damages red blood cells causing hemolysis, usually measured by the level of free-hemoglobin in plasma, where a concentration greater than 50 mg/dL is considered significant hemolysis (60). Shear stress can also damage white blood cells, platelets and proteins; and ADP released from damaged cells triggers platelet activation (61). In addition to surface shear stress described by the laminar flow model above, turbulent flow occurs throughout the ECLS circuit due to varied lumen diameters at different connection points, varied pressure, and changes in blood viscosity. Turbulent flow can cause blood trauma, hemolysis and is associated with a pro-coagulant response (62, 63).

In addition to shear-induced hemolysis that occurs when blood contacts the circuit wall and during turbulent flow, significant blood trauma and hemolysis can occur due to mechanical forces and negative pressures generated by the blood pump (64). The degree of hemolysis depends on the type of pump used, the pressure within the circuit and the number of revolutions per minute (rpm/s) needed to support the desired flow rate. Centrifugal pumps generally have a lower hemolysis index than peristaltic pumps when high flow rates (1-8 L/min) are used; however, when high rpm/s are required to support a low flow rate such as during neonatal ECLS therapy when small catheters, tubing and membranes create a low-flow, high-pressure circulation system, a roller pump is
generally preferred (60). This is because the blood contact time with the pump is increased, and red blood cell damage may occur at a faster rate with the centrifugal pump (60). The pump rpms are adjusted during circulation to prevent negative pressures of less than -650 mmHg in the venous line (line connecting the catheter outflow to the membrane inlet face), as significant hemolysis and sudden pressure drops inducing cavitation in the lines will occur (65).

2.4 Coagulation management during ECLS

To address thrombosis resulting from foreign surface exposure, shear stress and altered blood flow during ECLS, patients receive systemic anticoagulation therapy. Despite anticoagulant administration, the Extracorporeal Life Support Organization reported that significant clotting complications occurred in nearly 40% of all ECLS runs (60). A significant clotting complication was defined as a thrombotic occlusion that required replacement of a component of the ECLS circuit. The most common site of thrombus formation was the membrane. Additionally, circulation of damaged, pro-coagulant blood can result in systemic thrombosis which can be a lethal complication that often is unrecognized until postmortem assessment (60). For example, in a single-center prospective trial comparing clinical and autopsy findings of 78 adult ECMO patients over a 5 year period, deep venous thrombus formation occurred in 39.7% of patients, but was only clinically identified in 7.7% of patients (66). Arterial and end-organ thromboembolisms were found in 46.2% of patients but were clinically recognized in only 15.4% of patients.

The instance and severity of thrombotic complications presents a clear need for anticoagulation therapy; however, the task of achieving and maintaining therapeutic anticoagulation levels amidst constant blood stress from the extracorporeal circuit, frequent transfusions and underlying coagulopathies from initial injury is extremely challenging. There is currently no universal guideline for administration of anticoagulants or use of coagulation
monitoring tests for ECLS patients, resulting in highly variable, center-specific hemostasis management protocols (67, 68). Lack of consensus on the type of anticoagulant to use, the form of monitoring test to be employed and the therapeutic target range for each test, results in suboptimal outcomes and inconsistent patient care (67). The requirement for anticoagulation, in combination with consumption of platelets and clotting factors during circulation, leads to a high instance of bleeding complications, which occurs more frequently than thrombosis as described in Chapter 1 (69). In this final section summarizing the challenge of hemostasis during ECLS, we describe the clinically utilized anticoagulants for ECLS and introduce the concept of localized anticoagulation using biocompatible materials and surface coatings.

**2.4.1 Systemic anticoagulants for ECLS.**

**2.4.1.1 Unfractionated heparin.** At present, unfractionated heparin is the most consistently utilized anticoagulation method for ECLS and is considered the clinical standard (70). Heparin binds irreversibly to antithrombin III (ATIII), inducing a conformational change that increases ATIII inhibition of thrombin, Factor Xa and other clotting enzymes by 1000-fold compared to ATIII alone. Additionally, unfractionated heparin induces a 7-fold increase in release of TFPI from the endothelium, which inhibits factor Xa and the factor VIIa/TF complex as described in section 2.3.4.1. Heparin will not dissociate thrombin already bound to fibrin on material surfaces, and will not inactivate platelets (71). Yet, platelet activation and consumption are leading causes of bleeding complications in ECLS patients. Additionally, the activity of heparin is altered by the presence of plasma proteins; and it is neutralized by high-molecular weight von Willebrand factor multimers and platelet factor 4 (PF4), both released by activated platelets (72). Cumulatively, this leads to an unpredictable drug response from patient to patient. Heparin
induced thrombocytopenia (HIT) is an additional concern occurring in patients who develop antibodies against heparin-bound PF4. Heparin is metabolized by the liver and the reticuloendothelial system, with a half-life of approximately 60 minutes (73).

2.4.1.2 Direct thrombin inhibitors. Direct thrombin inhibitors (DTI), mainly bivalirudin and argatroban, were initially utilized in ECLS patients that developed HIT; but are now the anticoagulant of choice in certain centers due to more direct/linear pharmacokinetics (70). Unlike heparin, these agents do not require a cofactor, are not readily neutralized by plasma proteins, and can prevent thrombin induced platelet aggregation and inhibit thrombin already bound to fibrin clots (70, 72). Extensive use of these agents for ECLS across populations will require standardization of administration protocols and monitoring techniques, as well as improved understanding of potential antidotes to be used in case of overdose.

Bivalirudin is an analog of hirudin that binds both the active site and fibrinogen binding site of thrombin (74). It is metabolized by proteolytic cleavage (80%) or renal clearance (20%) and has a half-life of 25 minutes (60). Despite promising case reports, to date there are no large randomized prospective trials to provide thorough evidence for the role of bivalirudin anticoagulation during ECLS, and there are no Food and Drug Administration approved indications for pediatric populations (74-76). Further, there are no clear guidelines for optimal dosing and monitoring, and renal clearance may be problematic as many ECLS patients have altered renal function. For example, in a retrospective study bivalirudin dosing requirements increased by 75-125% in patients receiving extracorporeal renal replacement therapy (77).
Argatroban, a derivative of L-arginine, is a competitive thrombin inhibitor that reversibly binds the active site (73). It undergoes hepatic metabolism and has a half-life of 15 minutes (70). Like bivalirudin, there are no clear guidelines for optimal dosing and monitoring, and to date there are no prospective trials to demonstrate a clear therapeutic benefit during ECLS (70, 73). Both argatroban and bivalirudin are significantly more expensive in comparison to heparin; and both direct thrombin inhibitors lack a clear reversal agent (60, 73).

2.4.1.3 Anti-platelet agents. Antiplatelet agents such as acetylsalicylic acid (ASA) and prostacyclin have been utilized to supplement the use of unfractionated heparin or direct thrombin inhibitors for various forms of ECLS in small, single-center trials (70, 78). For example, ASA was used in combination with heparin in 15 patients receiving extracorporeal therapy for respiratory failure where no increased bleeding activity or transfusion demands were reported (79). ASA inhibits cyclo-oxygenase, reducing platelet production of thromboxane A₂ and inhibiting aggregation (70). Prostacyclin was used with heparin in children (80) and adults (81) receiving extracorporeal renal replacement therapy without adverse effects and shown to increase membrane life (time until replacement was necessary due to occlusion) in both cases versus patients that received heparin alone (82). Prostacyclin is an endogenous antithrombotic, described in section 2.3.4.6, that inhibits platelet aggregation by binding platelet receptors and inhibiting adenylyl cyclase activity. This leads to elevated cyclic adenosine monophosphate (cAMP) levels in the platelet, inhibiting multiple mechanisms of activation and aggregation (70).

2.4.2 Localized anticoagulation in the ECLS circuit. In addition to systemic anticoagulant drugs, local methods of anticoagulation have been introduced to inhibit clotting
within the circuit while preventing systemic bleeding complications. This includes regional infusion of anticoagulant drugs, such as citrate directly into the circuit (83), and development of anti-thrombogenic circuit surface coatings which will be discussed in depth in the next chapter (84). Regional citrate infusion is successfully utilized for ECLS application with low-flow requirements such as extracorporeal hemofiltration; however, it is insufficient for the high flow rates needed to support cardiopulmonary function (70). The leading surface coating strategy that has passed the developmental stage and is utilized in clinical practice involves immobilization of heparin on ECLS circuit surfaces. Immobilized heparin coatings are utilized for most current generation ECLS devices (e.g. Bioline®, Carmeda®); however, these coatings alone are not robust enough to prevent thrombus formation for an extended period, and supplementary use of systemic anticoagulants remains clinical practice (45, 60, 85). Both laboratory investigations and clinical studies have cautioned against the long-term stability of immobilized heparin coatings alone, or when used with low-dose heparin administration, as with extended use accumulation of fibrin and cellular deposits occurs, impairing gas exchange efficiency and causing resistance to blood flow (86-88). Additional studies caution against the use of heparin coated devices when HIT is suspected, as the HIT immune reaction has been observed to continue even after withdrawal of systemic heparin, implying potential heparin leakage from the circuit surface (89).

2.5 Conclusions.

Despite improvements in circuit design and biocompatibility of circuit materials, contact of blood with polymers in extracorporeal circulation systems causes extensive stress, thrombosis, hemolysis and consumptive coagulopathy. The high instance of both thrombotic and bleeding complications reported for ECLS demonstrates that current methods of coagulation management using systemic anticoagulant drugs and heparin-coated circuits are insufficient. The complexity
and delicate balance of coagulation management in the vascular endothelium suggests that a localized, multi-faceted approach to prevent coagulopathy during ECLS may be necessary. Understanding the factors defined by Virchow’s triad that determine blood-surface interactions is key for development of biocompatible materials to reduce coagulopathy. In the next chapter we discuss how scientists, clinicians, chemists and engineers are collaborating to create biocompatible materials modelled after the endothelium to provide a localized, efficacious solution to the challenge of coagulation during ECLS.

2.6 References.


Chapter Three:
Biomaterials for extracorporeal life support

3.1 Abstract.

Extracorporeal life support (ECLS) devices are utilized for diverse applications to include acute support of neonates and children with congenital abnormalities, emergency support for trauma-induced organ failure, transitional support for bridge to organ transplant and chronic support for terminal organ failure. The major complication that limits use of these life-saving devices across all disciplines is thrombosis, bleeding and inflammation caused by interactions of the blood with the foreign-surfaces of the devices. To address this challenge, trans-disciplinary efforts by scientists and clinicians look to the vascular endothelium as inspiration for development of biocompatible materials for ECLS. Here we describe clinically approved and new investigational biomaterial solutions for thrombosis such as immobilized heparin, nitric oxide-generating polymers, slippery non-adhesive coatings, and surface endothelialization. We describe how hemocompatible materials could abrogate the use of anticoagulant drugs during ECLS; and discuss special considerations for development of biomaterials specifically for ECLS to include preserving function of the artificial organ, longevity of use, and multifaceted approaches for the diversity of applications and functions of the devices.

3.2 Introduction.

Extracorporeal life support (ECLS) therapy has evolved significantly since initial use in 1953 by Dr. John H. Gibbon, Jr. to provide 26 minutes of heart and lung support during surgical repair of a cardiac defect (1). This initial device weighed over 1,000 kg and required multiple
technicians and specialists to operate. Since this time, ECLS technology has become significantly safer and less invasive, and has diversified to include capabilities for multi-organ failure including cardiac, pulmonary, renal and hepatic support. ECLS is now commonly performed outside of surgical suites, often in the intensive care unit, and can be utilized for weeks and even months of support (2, 3). This extended duration of use has allowed for ECLS as a bridge-to-transplant therapy (4). Further, ECLS has been used out of hospital during inter-hospital transport of unstable patients (5, 6); and has been utilized by the military in combat hospitals and during transcontinental aeromedical transport of combat casualties (7-9). Future applications include destination therapies for chronic disease using miniaturized, wearable ECLS devices that could provide support out of hospital in the patient’s home (10).

Despite the range of applications and patient populations for which ECLS has been utilized, the major challenge that limits use and poses a safety threat to the patient is disturbance of coagulation. The large surface area of interaction between blood and internal components of ECLS devices subjects the circulating blood to various detrimental effects. (11). The result is both thrombotic and hemorrhagic complications, which can often coexist simultaneously in the same patient (12, 13). When blood contacts the foreign surfaces in the ECLS circuits, adsorption of plasma proteins rapidly occurs (14). Platelets exposed to the proteins become activated, adhere to the surface of the circuit and release procoagulant factors to recruit and activate other platelets (15). Platelet and contact pathway activation initiates the coagulation cascade, leading to thrombin generation and conversion of fibrinogen to insoluble fibrin, stabilizing the clot (Figure 3.1) (16). Circulating red blood cells, platelets and leukocytes continuously become activated with exposure to the developing thrombus, causing release of soluble pro-inflammatory mediators and activation
Figure 3.1 Illustration of thrombus deposition on foreign material surface. The process is initiated by protein adsorption, followed by contact activation and platelet adhesion/activation. Coagulation activation leads to downstream activation of the complement system and inflammatory reactions. Reproduced with permission of ELSEVIER BV in the format Thesis/Dissertation via Copyright Clearance Center. Biran R, Pond D. Heparin coating for improving blood compatibility of medical devices. Advanced Drug Delivery Reviews. 2017; 112: 12-23.
of complement factors (14, 17). As the activated blood is returned to the systemic circulation, tissue factor release and pro-inflammatory factor expression occur within the endothelium (14, 18, 19). In addition to foreign surface contact, turbulent blood flow results from variability in the size of catheter and circuit tubing lumens. Pressure drops, shear stress and turbulence are generated by the blood pump and result in hemolysis, introduction of procoagulant microbubbles, endothelial collagen exposure and von Willebrand factor release from damaged cells (17, 20). Cumulatively, this causes severe risk of thrombus formation in the circuit and the patient, requiring administration of blood thinners to all patients receiving this therapy.

Simultaneous with thrombus formation, the hypercoagulable state that results from constant activation of blood and immune cells causes consumption of platelets and clotting factors, resulting in a frequently unrecognized hypocoaguable state and significant bleeding complications (21). Additionally, ECLS patients require continuous systemic anticoagulation therapy to prevent circuit thrombosis, occlusion and systemic thrombus formation; however, maintaining therapeutic anticoagulation is exceptionally challenging due to continuous blood trauma, high transfusion requirements and underlying coagulopathies resulting from the initial injury (22). Because of this, iatrogenic bleeding (secondary to anticoagulant administration and blood stress from circulation) is one of the most common complications associated with ECLS therapy and has been cited as problematic for >30% of patients, with recent reports suggesting this may occur in as many as 60-80% of patients (23-27). Anticoagulation for ECLS is typically performed with unfractionated heparin, direct thrombin inhibitors or combinations thereof; however, there are no universal guidelines for administration of these agents resulting in highly variable, center-specific hemostasis management protocols (22, 28). Lack of consensus on the type of anticoagulant to use,
the form of monitoring test to be employed and the therapeutic target range for each test, results in suboptimal outcomes and inconsistent patient care (28).

To address this challenge, research groups and industry partners are developing biocompatible surface coatings to be applied to the ECLS circuit, providing localized anticoagulation at the blood-biomaterial interface. Inspiration for these coatings is derived from the vascular endothelium, the endogenous blood-contacting interface that covers over 3,000-6,000 m² in adult humans (19, 29). The vascular endothelium serves as more than a passive barrier that separates blood from foreign sources of activation. The endothelium actively regulates blood fluidity and hemostasis by continuously modifying surface chemistry, excreted factors, flow distribution and permeability (29, 30). Further, the surface chemistry and functional characteristics of the endothelium are extremely heterogenous, varied to support tissue-specific needs and fluctuating metabolic demands (31). The intricacy and adaptability of the endothelium emphasize the complexity of developing an artificial hemocompatible surface.

Blood-biomaterial interactions during ECLS are dependent on several factors, to include the composition and surface characteristics of the material, the rate and path of blood flow, the duration of exposure, and the status and composition of the blood (32). These factors vary depending on the type of organ support the device is designed to supply, and the specific configuration of the circuit - which varies by manufacturer. For this reason, a universally hemocompatible material currently does not exist; and further, a material that is biocompatible for one ECLS application may not be compatible for another. In this review, we discuss hemocompatible materials that are clinically utilized for ECLS, including the biological inspiration that led to their development and the clinical and pre-clinical studies demonstrating their efficacy. It is important to note that to date administration of systemic anticoagulants is standard practice
for all forms of ECLS, regardless of biocompatible coatings, as current surface coatings alone are insufficient to prevent coagulation disturbances (33, 34). We will also discuss investigational surface coatings for ECLS and detail how these coatings are tested and optimized in the pre-clinical development phase. Finally, we will highlight key considerations for development of biomaterials specifically for ECLS, emphasizing current gaps in how materials are tested in the laboratory versus how they are utilized in the field.

3.3 Biomaterials for ECLS – coatings in the clinic.

There are many important considerations when developing a biomaterial for ECLS, as the devices vary significantly depending on the type of organ support and level of therapy they provide. In general, ECLS circuits consist of vascular access catheters, a blood pump, a membrane (modified for the specific type of organ support) and connective tubing. The circuits are generally composed of hydrophobic materials including polyvinylchloride (PVC), silicone, polycarbonate, polymethylpentene (PMP), polyurethane and/or polypropylene (11). Application of coatings over the surface of these polymers or incorporation of bioactive substances into the polymer matrix could alter the physical and mechanical properties of the polymer, potentially altering performance of the device. Additionally, the intended blood flow rates the devices are designed to support vary significantly depending on the application – for example, a rate of 3 mL/kg/min is recommended for adult respiratory support; whereas, a rate of 60mL/kg/min is recommended for adult cardiopulmonary support (35). The size of the devices is also an important consideration, as the devices are modified to accommodate neonates, pediatrics and adult populations. Finally, the duration of use of the device must be considered to ensure that the coating is functional for the time of intended use. These important considerations are integral in development of biomaterials for ECLS and will be discussed in context of the clinically available coatings described below.
3.3.1 Heparin. Immobilized heparin, first described in 1963 by Dr. Vincent Gott, is one of the first biomaterials applied to ECLS and is currently the most widely utilized (36). Heparin binds to and accelerates the activity of antithrombin III (ATIII), an enzyme that inhibits thrombin, Factor Xa and other clotting enzymes, and also performs numerous anti-inflammatory functions (37, 38). Within the vascular endothelium, heparin sulfate residues with heparin-like activity are anchored to the endothelium by a proteoglycan core (37, 39). These residues bind and localize ATIII on the endothelial surface (40) and are key components of the glycocalyx – a protective barrier between the blood and endothelial cells that performs mechanotransduction and micro-regulatory functions (39).

Heparin coatings (Figure 3.2 A) have been utilized clinically for over 4 decades, initially for cardiopulmonary bypass (CPB) and eventually for ECLS circuits and catheters (41). Clinical trials and meta-analyses comparing heparin-coated circuits to uncoated circuits for both CPB and ECLS showed that heparin coating reduced transfusion requirements (42-45), inflammatory cell activation (44, 46), complement activation (47, 48) and ICU length of stay (42-45); however, platelet count and activation were generally equivalent between heparin-coated and uncoated circuits (49, 50). Studies that distinguished between covalently bound versus ionically bound heparin found that covalent binding improved patient outcomes (42, 47), likely due to better retention of the heparin molecule which can be displaced and eluted from the surface via ion exchange (11).

While the above approach has made a significant impact on medical care, most reported benefits have been observed with short-term (~6 hrs) use (11). This may be sufficient for CPB, but ECLS can be continued for weeks to months at a time and requires a more robust solution. Currently no data supports utilization of immobilized heparin for ECLS without supplemental
Figure 3.2 Illustration of clinically available biomaterial coatings for extracorporeal life support. A) Immobilized heparin coating shown in green. Antithrombin III (ATIII) binds to heparin inducing a conformational change in ATIII, increasing enzymatic activity by 1,000-fold. ATIII binds and inactivates thrombin, inhibiting thrombus formation. B) Immobilized albumin competitively inhibits adsorption of procoagulant proteins like fibrinogen, providing surface passivation. C) Phosphorylcholine zwitterion coating modifies surface hydrophobicity and resembles the surface of cell membranes.
anticoagulation (51). In fact, even when used in combination with systemic anticoagulant drugs, this approach may be insufficient. For example, Lehle and colleagues examined heparin-coated membrane oxygenators from 28 ECLS patients receiving ECLS for respiratory failure for mean duration of 11 days with supplemental continuous heparin infusion (52). They observed significant thrombus formation and cellular deposits that formed “pseudomembranous” layers of 30-45 µm (primary wall thickness of gas exchange fiber is 75 µm). These layers can impede gas exchange, and in this study led to complete occlusion of 3 membranes, requiring circuit exchange during treatment. Pre-clinical comparison of heparin-coated ECLS circuits used with and without continuous heparin administration in an ovine model of oleic acid-induced lung injury demonstrated no difference in platelet aggregation and activation, thromboelastography or coagulation factors throughout 10 hours of circulation (53); however, after 10 hours greater thrombus deposition was observed on membranes from the group that did not receive continuous heparin infusion (54). Clinically, heparin-coated circuits have been utilized without systemic heparin (or with low-dose heparin protocols) for hours to days in trauma patients with bleeding complications; however, these patients are transitioned to heparin infusion protocols once bleeding is resolved (55-57).

In addition to insufficient longevity and efficacy, other critical limitations to this approach for ECLS are apparent. Heparin will not dissociate thrombin already bound to fibrin on material surfaces and will not inactivate platelets, meaning once a clot has formed heparin will not dissolve it (14). Additionally, extended exposure to heparin can invoke generation of antibodies against platelet activating factor 4 complexed with heparin, causing pronounced platelet activation in a condition called heparin induced thrombocytopenia (HIT) (51). This condition is normally treated
by discontinuation of heparin therapy; however, when a tethered-heparin circuit is in use, this may require complete withdrawal and replacement of the circuit (58).

3.3.2 Albumin. Introduced in the 1980s, immobilized albumin has been applied to ECLS materials and is most often used clinically when HIT is suspected (59, 60). In the vasculature, albumin is incorporated in the endothelial glycocalyx via interactions with proteoglycans and glycoproteins where it serves an important role in controlling the charge and permeability of the glycocalyx (39, 61). Albumin is the most abundant plasma protein and is thought to be inert to platelet activation and pro-thrombotic reactions when adsorbed on a biomaterial surface (62, 63). This is because albumin lacks specific binding sequences for platelets, leukocytes and coagulation enzymes (64). In this way, immobilized albumin acts as a “passivating agent” or competitive inhibitor of pro-coagulant plasma proteins – primarily fibrinogen, but also vWF, fibronectin, vitronectin and immunoglobins (Figure 3.2 B) (62). In a 2-hour ex vivo extracorporeal circulation study, use of albumin coating reduced circulating plasma concentrations of activation markers of platelets, complement, coagulation and immune cells. Furthermore, membranes had reduced fibrinogen adsorption at the end of study compared to uncoated controls (65). While albumin coated ECLS circuits have been successfully utilized in the clinic, primarily in the instance of HIT (66, 67), specific data demonstrating efficacy and comparing this approach to other coatings is lacking.

3.3.3 Phosphorylcholine (PC). PC is a neutral, zwitterionic phospholipid that composes the surface of the lipid bilayer of cell membranes. PC biomaterial coatings have been developed to modify the surface charge and hydrophilicity of foreign polymers to resemble that of endothelial cells (Figure 3.2 C) (11, 68). In addition to serving as a physical barrier, phospholipids play an important role in cell-to-cell communication through alteration in their charge and composition
For example, erythrocytes and platelets have asymmetric cell membranes, where in the native/resting state a greater proportion of neutral charge PC is exposed on the outer cytosolic leaflet and a greater proportion of negatively charged phosphatidylserine (PS) is present on the inner/cytoplasmic leaflet. When these cells become activated/pro-coagulant, phospholipid scrambling occurs, and negatively charged PS is exposed on the outer cytosolic leaflet at a greater proportion. In-vitro assessment of a PC coating applied to hollow-fiber oxygenator segments using ovine blood and 3 hour exposure time did not show an appreciable reduction in platelet deposition compared to heparin coating. When PC coating was compared to uncoated materials during CPB, the coating preserved platelet count, reduced platelet activation and reduced transfusion requirements; however, when compared to heparin and albumin-coated materials, PC-coating was similar and did not show additional benefit in terms of biocompatibility during use or in post-operative outcomes. One CPB study suggested that heparin and PC coatings may reduce inflammation when compared to albumin. Like albumin, while successful use of PC coatings during ECLS has been reported, there is no evidence to support use without continuous systemic anticoagulation therapy.

### 3.3.4 Other synthetic polymer surfaces
A number of synthetic polymer surfaces have been developed to increase hydrophilicity and biocompatibility of ECLS surfaces – these surfaces have hemocompatible properties but are not directly modelled after biological molecules. This includes poly(ethylene) oxide (PEO), poly 2-methoxyethylacrylate (PMEA) and surface modifying additives (SMAs). PEO, which alone has been shown to increase hydrophilicity and decrease protein adsorption, is incorporated in the Trillium® coating (Medtronic; Dublin, Ireland) which has a functional layer of PEO, sulfate/sulfonate groups and heparin. Applied to CPB, Trillium® was comparable to other heparin-coated materials and preserved platelet
count (81, 83), reduced platelet and granulocyte activation (80), and reduced complement activation versus uncoated controls (83). PMEA is generated on PVC and polypropylene via plasma activation (11) and is composed of a hydrophobic backbone along the polymer surface and a weakly hydrophilic surface on the blood-contacting face (84). The hydrophilic face attracts a thin boundary layer of water that prevents adsorption and deformation of proteins (84). SMA, which can be blended into the base polymer or applied to the surface, is a triblock copolymer with alternating hydrophobic and hydrophilic regions thought to compete for platelet binding sites on adsorbed proteins (84). PMEA and SMA materials have demonstrated questionable efficacy in the CPB literature, with some authors reporting comparable results to heparin-coated circuits (85), others reporting modest preservation of platelet count (86) and/or decreased transfusion requirements compared to uncoated controls (87), and still others reporting no appreciable benefit (84, 88). Additionally, in a pediatric CPB study, investigators reported transient leukopenia, elevated respiratory quotient and C-reactive protein levels when using PMEA membranes versus heparin membranes, which may have caused post-operative systemic inflammatory respiratory syndrome (89). Use of these synthetic surfaces without supplemental anticoagulation therapy is not advised.

3.4 Biomaterials for ECLS: investigational coatings.

A multitude of hemocompatible surface coatings are under development and have been applied to catheters and tubing segments. Many of these coatings demonstrate promising qualities that could be beneficial if applied to ECLS materials; however, in this review we summarize only surfaces that have reached a developmental stage where they have minimally been evaluated in a flow model.
3.4.1 Slippery, non-adhesive surfaces. One novel approach to inhibit protein adsorption and thrombus formation is utilization of liquid lubricant layers that have slippery, “omniphobic” characteristics. One such coating is tethered liquid perfluorocarbon (TLP), which is a bilayer coating that utilizes 1) a covalently bound flexible perfluorocarbon tether layer applied to the substrate/native polymer and 2) a thin, mobile liquid surface layer of perfluorocarbon lubricant (90). The tether layer attracts and anchors the liquid lubricant, preventing washout into overlaying fluid under flow. TLP is a modification of a coating approach called “SLIPS” or “slippery, liquid-infused, porous surfaces.” SLIPS were inspired by the Nepenthes pitcher plant which has a roughened, porous rim that attracts a liquid water layer via capillary forces to create a slippery surface that prevents insect attachment, causing them to slide into the pitcher of the carnivorous plant (91). Leslie, et al showed that TLP applied to acrylic and polysulfone reduces fibrinogen adsorption and platelet adhesion following incubation in whole blood (90). In vivo they examined TLP using an 8 hour porcine AV-shunt model and demonstrated that TLP-coated PVC tubing maintains patency under maximum flow rate of 300 mL/min without administration of heparin; whereas, four of five uncoated control tubing segments became occluded (90). Interestingly, they also found that TLP reduced biofilm formation 8-fold when applied to PVC versus uncoated PVC when Pseudomonas aeruginosa bacteria were grown on the surface for 8 weeks.

A limitation to the aforementioned studies is that in vitro tests were performed for 0.5 - 1.5 hours and in vivo assessment was conducted for 8 hours, so it remains unclear whether the liquid layer is retained on the material surface at a functional level beyond this time. Howell, et al showed that similar immobilized perfluorinated lubricant layers were stable following 16 hours circulation using deionized water at dialysis-like flow rates of 10-90 mL/hr (92). These studies also only evaluate TLP applied to tubing, without assessing the feasibility or functional impact of applying
the coating to ECLS membranes, which is the most common location where clotting occurs. Liquid lubricant layers have been applied to ECLS circuit materials using other methods, such as chemical vapor deposition of a perfluorinated organosilane on coronary catheters (93) and swelling of silicone oil into the polymer matrix of silicone tubing (94); however these materials have yet to be evaluated under flow conditions that replicate clinical ECLS conditions.

3.4.2 Nitric oxide (NO) releasing materials. Another bioinspired approach to prevent foreign surface mediated thrombosis during ECLS is development of NO-releasing materials that produce NO at the blood-biomaterial interface at a similar flux as occurs from healthy endothelial cells in the vasculature, estimated to be 0.5-4 x 10^{-10} mol cm^{-2} min^{-1} (95). In the endothelium NO is a potent vasodilator, prevents platelet aggregation and adhesion, inhibits monocyte activation, and also has bactericidal and antimicrobial properties (96-98). Various NO donor species such as diazeniumdiolated dibutylhexanediamine (DBHD/N2O2) (99-101) and S-nitroso-N-acetylpenicillamine (102-104) have exhibited anithrombogenic properties when incorporated into medical polymers and evaluated in a 4-hour extracorporeal circulation model in rabbits. In this model, the coatings are applied to PVC tubing that forms an arterio-venous shunt when connected to the carotid artery and jugular vein via two angiocatheters. Thrombus deposition area, platelet count and activity (platelet P-selectin expression and aggregometry) and plasma clotting time are assessed (105). To investigate the NO-release capacity of these materials in vivo beyond the 4-hour circulation time frame, Brisbois and colleagues evaluated cannulas fabricated from SNAP-doped Elast-eon™ E2As polymer following implantation in the jugular veins of sheep for 7 days (106). Thrombus area was reduced in the SNAP-doped group, and bacterial adhesion was reduced by 90% compared to controls. Additionally, at the end of use, NO flux from the SNAP-doped catheters was 0.6 ± 0.3 x 10^{-10} mol cm^{-2} min^{-1}, still within the range of endothelial NO-flux. A
limitation to this approach is that only a finite amount of NO donors can be incorporated into the polymer matrix; and the longevity and efficacy of this approach remains to be evaluated using flow rates and study durations that simulate the clinical condition for specific forms of ECLS.

Another solution to extend performance of NO-releasing materials is utilization of a nitric oxide catalyst to generate NO from bio-available NO-donors, such as S-nitroshothiols (RSNOs) (107). Metal-organic frameworks (MOFs) such as the copper-based H₃[(Cu₄Cl)₃-(BTTri)₈] (H₃BTTri = 1,3,5-tris(H-1,2,3-triazol-5-yl)benzene), or CuBTTri, are capable of generating NO from endogenous sources to provide a continuous source of NO as long as bioavailable donors are present - rather than pre-loading circuit materials with finite levels of synthetic donors (107). CuBTTri accelerated NO release from S-nitrosoglutathione (GSNO) nearly 65-fold relative to thermal decomposition of GSNO (108) and CuBTTri impregnated polymers have shown significant NO release from a variety of RSNOs (107-109). CuBTTri polymers have yet to be evaluated in vivo under flow. In addition to copper-based MOFs, NO-generating copper nanoparticles have been incorporated into polyurethane and applied to PVC tubing and angiocatheters for evaluation in the 4-hour rabbit circulation model (110). The coating preserved platelet count and function, reduced thrombus area and fibrinogen Aγ dimer formation and prevented monocyte activation, so long as a continuous infusion of an exogenous RSNO was used in combination with the coating. These effects were not observed without supplemental RSNO administration, suggesting endogenous RSNO concentration may be insufficient for this approach. Combination coatings that incorporate both NO-generating copper nanoparticles with a synthetic donor (SNAP) into a polymer composite produce NO-flux as high as 11.7 ± 3.6 x 10⁻¹⁰ mol cm⁻² min⁻¹, causing a 99.8% reduction in bacterial adhesion and 92% reduction in platelet adhesion in
vitro compared to controls (111). This approach requires further evaluation in vivo using ECLS-relevant flow conditions.

3.4.3 Immobilized direct thrombin inhibitors (DTIs). Similar to immobilization of heparin, immobilized DTIs like bivalirudin and argatroban could prevent thrombus formation at the biomaterial surface, while reducing systemic complications. Unlike heparin, these agents do not require a cofactor and can inhibit thrombin already bound to fibrin clots (112). For example, argatroban has been applied to polyurethane angiocatheters and PVC tubing and evaluated in vivo in the 4-hour rabbit arterio-venous shunt model described above (113) with blood flow rate ranging from 59-80 mL/min. The argatroban coating reduced the total area of thrombus formation on the tubing but did not preserve platelet count, as argatroban does not inhibit platelet activation. Thrombin clotting time in circulating blood was elevated in the argatroban group, suggesting some leaching may occur (113). The efficacy and longevity of these materials at flow rates typically observed during ECLS (0.5-7 L/min) have yet to be assessed, and application of the coating to the membrane will require further investigation.

3.4.4 Glycosaminoglycan polymer brushes. The endothelial glycocalyx consists of an extensive layer of glycosaminoglycan containing proteoglycans organized into “bush-like” structures of 100-200 nm that provide a physical barrier to protein adsorption. Attempts to recreate organized glycosaminoglycan-rich structures is challenged by the fact that these residues are strongly polyanionic, making assembly electrostatically unfavorable (114). To overcome this, a technique was developed to form polyelectrolyte multilayers that couple polycations like chitosan with polyanions like hyaluronan (115). This technique was used to form polymer brushes rich with sulfated glycosaminoglycans found in the glycocalyx, organized into structural domains as they are in the endothelium. The materials successfully inhibited protein adsorption and fibrin
formation but require further evaluation in vitro and under ECLS-relevant flow conditions (114). Additionally, this approach has been coupled with nitric oxide releasing donor species to combine multiple thrombo-regulatory mechanisms as occurs in the endothelium (116).

3.4.5 Factor XIIa inhibitor. Coagulation fXIIa is instrumental in thrombosis during blood exposure to foreign surfaces, initiating contact pathway activation (62). To target this, application of corn trypsin inhibitor (CTI), a selective fXIIa inhibitor, was applied to polyurethane catheters using polyethylene glycol (PEG) conjugation to immobilize CTI on the catheter surface (117). These catheters were evaluated in a rabbit model where time to occlusion was extended 2.5-fold compared to uncoated controls. There was no difference in prothrombin time or activated partial thromboplastin time from circulating blood in either group, suggesting that CTI does not leach from the material surface. Others reported reduced fibrinogen adsorption using PEG conjugated CTI on polyurethane following incubation in plasma (118). Further studies are needed to evaluate this approach applied to other circuit components under flow conditions and exposure times that resemble ECLS.

3.4.6 Endothelial cell seeding. Rather than replicate a specific thrombo-regulatory aspect of the endothelium, the idea of applying a layer of endothelial cells over the surface of medical polymers to achieve a completely hemocompatible surface has been investigated. This is accomplished by seeding cells onto the surface directly (in vitro pre-endothelialization) or by formulating a surface that will promote attachment and differentiation of circulating endothelial progenitor cells on the biomaterial surface to self-assemble an endothelial layer in vivo (in situ endothelialization (119). The pre-endothelialization approach, which has been utilized for vascular grafts and stents, is now being adapted for ECLS applications. For example, Pflaum, et al applied endothelial cells isolated from human umbilical cord blood to segments of polymethyl pentene
(PMP) from a hollow-fiber gas exchange membrane for ECLS (120). Because endothelial cells will not adhere to a strongly hydrophobic surface like PMP, a layer of titanium dioxide was applied to allow for cell seeding; however, the titanium dioxide coating alone reduced the oxygen transfer rate of the fibers by 22% compared to controls, prior to addition of the endothelial cell layer. Endothelial cells adhered to the titanium dioxide coated fibers forming a monolayer, and withstood shear stress at physiological levels (30 dyn/cm²). Additionally, the seeded endothelial cells remained in a non-activated state, determined by expression of pro-inflammatory/prothrombotic markers (ICAM-1, VCMA-1, E-selectin, tissue factor, thrombomodulin). While this is a provocative approach, the feasibility of manufacturing such a product – including shelf-life, sterilization, cell source, cost, etc., and the ability to apply the cells to complete components of the ECLS circuit rather than small membrane segments tested thus far must be established.

3.5 Conclusions.

The vast number of strategies aimed to develop a hemocompatible surface for ECLS emphasizes the importance and complexity of this problem. After reviewing the limitations of clinically available coatings and understanding how investigational coatings are assessed, it is apparent that there is a disconnect between how these coatings are utilized in clinic and how coatings are developed and optimized in the lab. For example, while it is important and feasible to assess novel coatings using stationary laboratory assays in whole blood or plasma during the initial optimization phase, it is necessary to establish if a coating will perform with similar efficacy and durability under in vivo flow conditions in animal models that closely resemble the physiologic complexity of a patient and account for the intricacies of patient care. Use of arterio-venous shunt models in small animals does not generate flow rates that would be utilized for most forms of ECLS, and also does not account for hemolysis and shear induced by the blood pump.
Additionally, the majority of studies evaluate coatings applied to tubing only, while the membrane is the most common site of clotting complications (34). This is likely the most challenging component of the circuit to coat due to the large surface area, complex structure and possibility of altering performance of the native polymer – such as gas transfer and permeability of membrane fibers designed for extracorporeal pulmonary support. The condition of the blood in investigational studies is also very important as injury status, species, hydration, anesthesia medications, anticoagulation drugs, presence of bacteria etc. will alter the coagulation response of the blood. Finally, and potentially most importantly, many of the studies mentioned in this review were conducted for only a few hours, as many coatings for ECLS were initially developed and evaluated for cardiopulmonary bypass utilizing a 6-hour time frame. Studies of extended duration specific to certain ECLS applications are necessary to ensure that the biomaterials can perform for the duration of intended use. For example, the average duration of ECLS for adult respiratory support is 271 hours, which is more than 45 times the current 6-hour testing protocol (3). In summary, it is important for biomedical scientists, chemists, engineers, clinicians and industry to work together to understand the complexity of clinical care and patient management, the fundamentals and properties of materials science, the intricacies of coagulation and thrombogenesis, and the challenges of developing and manufacturing a medical product. The future of ECLS will depend on these types of interactions to develop a robust solution for ECLS without anticoagulation.

3.6 References.


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Chapter Four:

A metal organic framework reduces thrombus formation and platelet aggregation ex vivo

4.1 Abstract.

4.1.1 Background. Management of hemostasis is a key challenge during extracorporeal life support (ECLS). Metal organic frameworks are being investigated for use as nitric oxide (NO) catalysts for incorporation into ECLS circuitry to prevent thrombosis at the blood-biomaterial interface. A specific metal organic framework, CuBTTri, has been shown to accelerate NO-release from bioavailable donors like S-nitrosoglutathione (GSNO). We hypothesized that CuBTTri would reduce thrombus formation in whole blood (WB) and inhibit platelet aggregation.

4.1.2 Methods. CuBTTri particles were added to WB and analyzed by thromboelastography (TEG). Biostable metal-based frameworks (MIL-100, Zeolite USY) were added to blood as controls, in addition to a saline vehicle control. Reaction time (R), clot formation time (K), alpha-angle, clot strength (MA), and percent fibrinolysis (LY30/LY60) were recorded. The effect of CuBTTri on platelet aggregation was assessed in WB and platelet rich plasma (PRP), both with and without addition of GSNO.

4.1.3 Results. CuBTTri significantly prolonged R and K and decreased alpha-angle and MA relative to the metal framework controls. Dose escalation results suggest that the control metal-based particles induce thrombus formation, as R and K were significantly reduced.

Portions of this chapter were previously published and have been used with permission from the publisher. Roberts TR, Neufeld MJ, Meledeo MA, Cap AP, Reynolds MM, Cancio LC, Batchinsky, AI. A metal organic framework reduces thrombus formation and platelet aggregation ex vivo. Journ of Trauma and Acute Care Surg. 2018;85(3):572-579.
compared to the saline control; however, this did not occur in the CuBTTri group. LY30/LY60 were elevated in the CuBTTri group versus saline (p = 0.014) but were not different from metal framework controls. CuBTTri alone and with GSNO reduced platelet aggregation in WB (p <0.0001), while GSNO alone had no effect. In PRP, GSNO and CuBTTri inhibited platelet aggregation separately, and together decreased aggregation by 35% relative to GSNO alone (p = 0.004).

4.1.4 Conclusions. CuBTTri reduced thrombus formation and inhibited platelet aggregation. CuBTTri enhanced platelet inhibition with GSNO, which was consistent with reports that CuBTTri accelerates NO release from endogenous NO-donors. This initial characterization of CuBTTri demonstrated its potential as an anti-thrombogenic agent to be further evaluated with incorporation into ECLS circuitry.

4.2 Background.

The use of extracorporeal life support (ECLS) is increasing in trauma populations for severe cardiopulmonary failure, as advancements in device circuitry and ease of cannulation have improved feasibility (1). Trauma patients are optimal candidates for ECLS as they are generally young and may have a reversible disease process without co-morbidities (2). In a systematic review of ECLS for trauma from 1994-2015, survival to discharge ranged from 50-79%, demonstrating the potential of this therapy in trauma populations (3). Additionally, data from recent military conflicts show that a large number of casualties incur combined injuries of increasing severity which necessitates utilization of various forms of ECLS (4, 5). Several forms of ECLS have been used in combat zones and include renal support, extracorporeal membrane oxygenation (ECMO) and combinations thereof (4, 6).
Despite technological advances, bleeding remains a major ECLS complication, often requiring surgical intervention and extensive transfusion. At the same time, systemic anticoagulation is recommended for all current forms of ECLS to minimize thrombosis (7). Both thrombotic and hemorrhagic complications take place during ECLS, resulting from blood contact with foreign polymers in the circuitry, platelet loss, consumption of clotting factors, hemodilution, tubing size to membrane surface ratio and shear-stress (8, 9). Use of systemic anticoagulants, while potentially mitigating thrombosis, increases risk of bleeding (10, 11). Systemic anticoagulation with unfractionated heparin (UFH), is the leading clinical strategy to prevent thrombotic complications with ECLS (7); however, no universal guideline for heparin administration or consensus on coagulation monitoring exists outside of variable, center-specific management protocols (8, 12). In summary, thrombotic and bleeding complications present challenges to management of ECLS and limit its utilization in trauma patients due to ongoing bleeding risk.

Several research efforts investigated localized, circuit-focused anticoagulation strategies, including regional infusion of anticoagulants such as citrate directly into the ECLS circuit (13) and development of anti-thrombogenic surfaces (5, 11). Some examples of modified surfaces include tethering heparin or plasma proteins to the circuit surface (14-16), imparting hydrophilic or omniphobic characteristics on circuit materials (17-19) and development of biomimetic materials inspired by the endogenous endothelium, such as nitric oxide (NO) releasing materials (20-22). Of these efforts, NO, is a promising therapeutic candidate as an endogenous substance with a short half-life, reversible action on platelets and localized action at the material and blood interface (22).

A new approach to anticoagulation is utilization of an additive that allows for enhanced NO generation from endogenous NO donors within the body. Metal-organic frameworks (MOFs) such as the copper-based $\text{H}_3[(\text{Cu}_4\text{Cl})_3-(\text{BTTri})_8]$ (H$_3$BTTri = 1,3,5-tris(1H-1,2,3-triazol-5-
yl)benzene), or CuBTTri, are capable of generating NO from NO donors, such as \( S \)-nitrosothiols (RSNOs), which are biologically available in the blood (23). In this way, CuBTTri serves as an NO catalyst, generating NO from endogenous sources rather than pre-loading circuit materials with finite levels of NO donors. Incorporation of NO producing MOFs like CuBTTri into blood contacting biomedical polymers could provide a continuous source of NO so long as endogenous donors are present (Figure 4.1 A). This may impart platelet quiescence at the blood and biomaterial interface without the untoward systemic effects of systemic anticoagulants. At present, CuBTTri is the most promising candidate for biomedical applications due to the excellent stability of this material in biological media, including blood (23). The ability of CuBTTri to generate NO from a variety of RSNOs has been confirmed for both free CuBTTri particles and upon incorporation into polymers (23-25). Whereas CuBTTri particles and the impregnated CuBTTri polymers enhanced NO generation from multiple different RSNOs, its effects in blood on real-time clot formation and platelet aggregation have not been investigated. The objective of this study was to test the anti-thrombogenic potential and bioactivity of CuBTTri using thromboelastography (TEG) (Figure 4.1 B) which has been used to investigate the impact of various biomaterials at the blood/biomaterial interface (24-26). Additionally, we investigate the effect of CuBTTri on platelets directly via platelet aggregometry, utilizing the bioavailable NO donor \( S \)-nitrosothiol (GSNO) as a positive control to demonstrate the catalytic activity of CuBTTri. We hypothesized that CuBTTri particles reduce clot strength and decrease platelet aggregation in ex-vivo human blood. Additionally, we hypothesized the CuBTTri would further reduce platelet aggregation in the presence of GSNO, in comparison to GSNO alone.
Figure 4.1 A) Metal organic framework (MOF) coated Tygon segment. The specific MOF is designed to generate nitric oxide (NO) from biologically available donors, such as S-nitrosothiols (RSNO), releasing a disulfide byproduct (RSSR). B) Schematic of nitric oxide catalysis by metal organic framework suspended in blood for thromboelastography (TEG) analysis.
4.3 Methods.

4.3.1 Preparation of MOF. CuBTTri was synthesized following a previously reported method (23). Briefly, 225 mg of H$_3$BTTri was added to 40 mL of DMF; the solution was then adjusted to a pH of 4 using hydrochloric acid to dissolve H$_3$BTTri into solution. Separately 335 mg of CuCl$_2$$\cdot$2H$_2$O was dissolved in 5 mL of DMF. The solution was then added to the H$_3$BTTri solution and placed in the oven at 100 °C for three days, during which crystal growth occurred resulting in the formation of a deep purple powder. Subsequently, upon solvent removal via centrifugation, the powder was rinsed several times with additional DMF followed by Millipore water (18 MΩ•cm). The rinsed CuBTTri was then added into a vial containing Millipore water and placed in the oven at 80°C for an additional day to allow for solvent exchange, resulting in a light purple powder. The MOF was then centrifuged to remove water and allowed to air dry for several days. Confirmation of CuBTTri was performed by powder x-ray diffraction on a Bruker D-8 Discover DaVinci X-ray diffractometer (Cu Kα X-ray source, line focus) (Bruker, Billerica, MA, USA). All measurements were performed at 4-350 with a step count of 1.0 (Appendix C).

4.3.2 Preparation of S-nitrosoglutathione. GSNO was prepared following an adapted method of a previously reported synthesis by Hart (27). In brief, glutathione (1.56g, 5 mmol) was added to a round bottom flask containing 8 mL of Millipore water and 2.5 mL of 2 M hydrochloric acid. Following addition of glutathione, the flask was placed in an ice bath and cooled to 0° C, to which sodium nitrite (0.342 g, 5 mmol) was added. The resulting mixture was covered, protected from light and allowed to stir for approximately 40 min. The red solution was then treated with 10 mL of ice-cold acetone and stirred for an additional 10 min. The resulting red precipitate was isolated using filtration and subsequently washed with 5 × 5 mL of ice-cold water, 3 × 5 mL ice-cold acetone, and 3 × 5 mL ice cold diethyl ether. The precipitate was then allowed to dry followed
by placement under vacuum to remove the presence of trace solvent and stored at -20°C in the absence of light. UV-vis spectroscopy was used to confirm GSNO formation in water by the appearance of peaks at 335nm (π → π*) and 545nm (nN → π*). A GSNO stock solution was prepared approximately 10 min prior to all runs by dissolving 10 mg GSNO in 10 mL saline prior to use on each study day. The resulting solution was then covered to protect from light and used within 3 hours of preparation.

4.3.3 Blood collection and preparation. Whole blood was collected in a de-identified fashion at the U.S. Army Institute of Surgical Research Blood Bank from a total of 20 healthy male and female donors between 18–40 years of age under an approved standard operating procedure at the U.S. Army Institute of Surgical Research Blood Bank. Donors were non-smokers with no known coagulopathies. Blood from the same donor was used for both TEG and platelet aggregometry, provided both experiments could be completed within 2 hours post-collection time (for TEG, n = 20; platelet aggregometry, n = 12). Due to the unprecedented nature of this work an effect size could not be estimated, and the sample size was chosen based on previous studies utilizing similar methods. Blood was collected in 3.8% citrated BD Biosciences vacutainers (Franklin Lakes, NJ, USA) and analyzed within 2 hours of collection time at 37°C. Platelet rich plasma (PRP) was prepared by centrifugation (200-g for 10 min without braking). PRP platelet counts were standardized to 250 x 10⁹/mL by dilution with the donor’s platelet-poor plasma (obtained by secondary centrifugation at 2000-g for 20 min without braking). Counts were determined using an ABX Micros 60 hematology cell counter (Horiba Medical; Irvine, CA, USA).

4.3.4 Preparation of MOF solutions. A CuBTTri solution was prepared by suspending CuBTTri in phosphate buffered saline (PBS), pH=7.4, to final concentrations of 2 mg / mL (CuBTTri High) and 0.2 mg / mL(CuBTTri Low). As controls, solutions of the same concentration
were prepared utilizing commercially available metal-based frameworks MIL-100 (Strem Chemicals, Inc.; Newburyport, MA, USA) and Zeolite USY (Zeolyst International; Conshohocken, PA, USA) to account for the effects of crystalline metallic particles in solution. Mil-100 is an iron 1,3,5-tricarboxylate based MOF, which has been utilized for drug carrying applications (28, 29). Zeolite USY is an aluminum-based inorganic framework commonly used for solid acid catalysis. These specific metal frameworks were chosen to serve as metal-framework variants of the CuBTTri as they remain highly stable in aqueous conditions and will not undergo substantial decomposition in blood.

4.3.5 Thromboelastography. For each donor (n = 20), 100 µL of each MOF solution (CuBTTri, Mil-100 and Zeolite) was added to 300 µL of citrated whole blood. For 10 donors, a high concentration of each MOF solution (2 mg/mL) was added, and for the remaining 10 donors, a low concentration (0.2 mg/mL) of MOF solution was utilized. As a vehicle control, 100 µL of PBS was added to 300 µL citrated whole blood. Each mixture was gently inverted, and 340 µL was transferred to a standard TEG clear cup to which 20 µL CaCl₂ had been added. TEG was performed between 20 – 30 min post-collection time without activating reagents using the TEG 5000 Hemostasis Analyzer System (Haemonetics Corporation; Braintree, MA, USA). The following variables of interest were recorded: reaction time (R), initial clot formation time (K), amplification rate (α-angle), clot strength (MA), and percent fibrinolysis at 30 min and 60 min after MA was detected (Ly30 and Ly60). Samples were run in duplicate for each treatment, and the average of the duplicates was taken for each donor. Duplicate TEG tracings that were inconsistent (i.e. instrument error, early splitting of TEG tracing) were excluded from analysis.

4.3.6 Platelet aggregometry. Collagen stimulated platelet aggregation was determined using impedance aggregometry (Multiplate 5.0, Verum Diagnostica; Munich, Germany). For each
donor (n = 12), 300 µL whole-blood (WB) was diluted with 200 µL of 3 mM CaCl$_2$ in 0.9% NaCl and incubated at 37° C for 3 min. 50 µL of CuBTTri solution (0.4 mg / mL saline) (CuBTTri Group and CuBTTri+GSNO Group), 50 µL Zeolite USY solution (0.4 mg / mL saline) (Zeolite Group) or 50 µL saline (Saline and GSNO groups) was added. Immediately following addition of MOF solution or saline, 50 µL GSNO in saline (50 µM) was added to the GSNO Group and CuBTTri+GSNO Group, and 50 µL saline was added to all other groups (Saline, CuBTTri and Zeolite Groups). Samples were incubated for 1.5 min, prior to addition of 20 µL collagen reagent (3.2 µg / mL) which initiated the 12 min data collection period. This process was repeated using platelet rich plasma (PRP) from the same donors. Per manufacturer’s instruction, if the internal control (duplicate sensors) was outside the acceptance range (≥ 20% difference), the result was excluded from analysis.

4.3.7 Statistical analysis. Statistical analysis was conducted using SAS 9.4 (Cary, North Carolina, USA). A two-tailed test was performed with an alpha = 0.05 for significance. Results are expressed as mean ± standard deviation. The distribution of the data was tested using a Shapiro-Wilk test. A mixed model with a random effect on donor was used to look for group differences. For the TEG results, between group comparisons were made separately for the low dose groups and high dose groups. The low and high doses were then compared within the same group. Tukey’s adjustment was made for multiple comparisons.

4.4 Results.

4.4.1 Thromboelastography. The metal-framework controls (Zeolite and Mil-100) had accelerated clot reaction (R) and formation times (K), as well as more rapid clot amplification rate ($\alpha$-angle) in comparison with the vehicle control (Figure 4.2). In the Mil-100 Group, this was significant at both the low and high dose for reaction time (low = 25% decrease, high = 48%
Figure 4.2 Figure represents mean ± standard deviation of the (A) reaction time, (B) clot formation time and (C) amplification angle determined by thromboelastography of human donor blood (n ≥ 6 / group) treated with free particles of a nitric-oxide catalyzing metal organic framework (CuBTTri). CuBTTri is compared to metal-based framework control particles (Zeolite and MIL-100) and phosphate buffered saline as a vehicle control (PBS). Particles were added at a low (0.2mg/mL) and high (2mg/mL) dose. Between group comparisons for particle additives were made for particles of the same dose (low dose additives compared separately from high dose). *Represents significant difference between groups (p < 0.05). **Represents significant difference between low dose and high dose within the same group (p < 0.05).
decrease) and amplification rate (low = 11% increase; high = 26% increase), where the percent change from the vehicle control was greater in the high dose group. In the Zeolite group, deviation from the vehicle control was significant in the high dose group (R = 32% decrease, K = 23% decrease, α-angle = 16% increase). This effect was not observed in the CuBTTri group, which did not differ from the vehicle control for clot reaction and formation time or amplification rate (Figure 4.2). Additionally, clot reaction and formation times were significantly longer in the CuBTTri group versus both the Zeolite and Mil-100 groups at both the low and high dose (Figure 4.2 A and B). Amplification rate was significantly reduced in the CuBTTri group in comparison with the Mil-100 Group at both doses and the high dose Zeolite Group (Figure 4.2 C). Clot strength was significantly reduced in the CuBTTri group versus the metal framework controls and the vehicle control in both the low and high dose groups (Figure 4.3 A). This reduction in clot strength was more pronounced in the high dose group, where CuBTTri exhibited an 8% decrease in comparison with both the Mil-100 and Zeolite group, and a 4% decrease from the vehicle control. LY30 and LY60 were significantly elevated in the high dose CuBTTri group relative to the vehicle control group but was not different from the Mil-100 or Zeolite Groups (Figure 4.3 B and 4.3 C). This difference was not observed in the low dose CuBTTri group.

There were no differences for any TEG variables between the low and high doses of the same additive (e.g. CuBTTri low vs CuBTTri high), with the exception that α-angle was significantly higher (+8°) in the high dose Mil-100 Group vs the low dose Mil-100 Group. There was a numerical trend suggesting a pro-coagulant response to elevated concentrations of the metal framework additives, as the high dose groups had shorter R and K times, as well as elevated α-angles and MA in comparison with the low dose groups.
Figure 4.3 Figure represents mean ± standard deviation of the (A) clot strength, (B) clot lysis (LY30%) and (C) clot lysis (LY60%) determined by thromboelastography of human donor blood (n ≥ 6 / group) treated with free particles of a nitric-oxide producing metal organic framework (CuBTTri). CuBTTri is compared to metal-based framework control particles (Zeolite and MIL-100) or phosphate buffered saline as a vehicle control (PBS). Particles were added at a low (0.2mg/mL) and high (2mg/mL) dose. Between group comparisons for particle additives were made for particles of the same dose (low dose additives compared separately from high dose). *Represents significant difference between groups (p < 0.05).
4.4.2 Platelet aggregation. In whole blood (Figure 4.4 A), CuBTTri reduced platelet aggregation compared to the Zeolite (22% decrease) and Saline (23% decrease) Groups. A similar reduction was observed in the CuBTTri+GSNO group (21% decrease vs. Zeolite; 22% decrease vs. Saline), and there was no difference between the CuBTTri and CuBTTri+GSNO groups. GSNO alone had no effect on platelet aggregation relative to the Zeolite and Saline Controls. In PRP (Figure 4.4 B), CuBTTri again inhibited aggregation in comparison to Zeolite (19% decrease) and Saline (21% decrease) Groups. GSNO alone significantly reduced platelet aggregation in comparison to the Saline (58% decrease), Zeolite (57% decrease) and CuBTTri (47% decrease) Groups. Aggregation was further inhibited (35% decrease) in the CuBTTri+GSNO group in comparison with the GSNO group.

4.5 Discussion.

ECLS has become more widely used since its efficacy was proven during the H1N1 pandemic (30, 31) and significant miniaturization, safety and design improvements in modern devices, many of which can be initiated percutaneously and with dialysis-like invasiveness (4, 7, 32, 33). The cumulative volume of ECLS cases for cardiac, respiratory and dialysis indications is ever increasing and the indications for ECLS use in trauma are expanding (3, 7). The primary hurdle to wider adoption of ECLS as a life-saving capability in severely injured patients is the unresolved problem of blood/polymer interactions in ECLS circuitry. This study investigated a new approach to anticoagulation management, which may have fundamental implications on how we manage patients undergoing ECLS. We utilized an ex vivo set-up and accepted coagulation monitoring assays to investigate local effects of a copper-based NO catalyst on whole blood and PRP. Our primary finding is that the MOF NO-catalyst CuBTTri weakened clot strength in whole blood and reduced platelet aggregation in both whole blood and PRP. CuBTTri also had a
Figure 4.4 Figure represents mean ± standard deviation of collagen-stimulated platelet aggregation measured by impedance aggregometry. Results expressed in aggregation units (AU). Aggregation was measured in (A) whole blood (n ≥ 10 / group) and (B) platelet-rich plasma with a standardized platelet count of 250 x 10^9/mL by dilution in donor’s platelet-poor plasma (n ≥ 10 / group). Prior to addition of collagen reagent, blood was incubated for 1.5 min with particles of a nitric-oxide catalyzing metal organic framework (CuBTTri). CuBTTri was compared to a biologically-stable metal-based framework control (Zeolite) and saline as a vehicle control (PBS). Immediately following addition of CuBTTri or controls, GSNO (50 µM) was added to blood directly (GSNO Group) and to blood containing CuBTTri particles (CuBTTri + GSNO Group). *Represents significant difference vs. Saline Control (p < 0.05). **Represents significant difference vs. Zeolite Control (p < 0.05). ***Represents significant difference vs. CuBTTri (p < 0.05). ****Represents significant difference vs. GSNO (p < 0.05).
potentiating effect on platelet inhibition in PRP when combined with the NO-donor GSNO. This 
ex vivo experiment suggests that incorporating CuBTTri into ECLS circuitry may reduce the need 
for systemic anticoagulation and reduce platelet consumption; further study is indicated.

Our TEG results demonstrate the specific antithrombogenic effects of CuBTTri in whole 
blood, relative to the other metal-based frameworks, used as controls. CuBTTri increased R and 
K and decreased α-angle and MA. This finding is consistent with TEG results reported previously 
in which the NO-donor Deethylamine NONOate (DEA/NO) was added to whole-blood and 
platelet rich plasma (34). In the aforementioned study, addition of increasing concentrations of the 
NO-donor (0.1 - 1.0 µM) prolonged the R time and decreased α-angle and MA when adenosine 
diphosphate and arachidonic acid were used to activate platelet membrane receptors (34). We did 
not observe a significant dose-dependent response to CuBTTri in this study; however, CuBTTri 
acts as a catalyst, so NO-derived effects may be limited by availability of endogenous NO-donors 
in the small blood volumes utilized in this study.

The dose escalation results in the control metal-framework groups (Zeolite and Mil-100) 
suggest that, in general, addition of metal-based framework particles causes a pro-coagulant 
response by reducing R and K and increasing α-angle relative to the vehicle control. This effect 
may be attributed to the particles serving as sites for plasma protein binding, activating platelets 
and inducing the intrinsic pathway, as has been reported in multiple studies investigating 
nanoparticle thrombogenicity (35). This response, however, was blunted in the CuBTTri Group. 
This suggests that CuBTTri has specific antithrombogenic properties that offset the prothrombotic 
effects of metal-based framework particles in blood. It is important to note that for biomedical 
applications, the intent is to immobilize CuBTTri within a polymer matrix of the ECLS circuitry 
rather than suspending particles into blood directly, - which alongside the ex-vivo nature of our
work are important limitations of our study. Embedding CuBTTri into the ECLS circuitry polymers will eliminate the procoagulant effects of metal framework particles suspended directly in blood as observed here, but also may modify the interaction of the CuBTTri NO catalyst with endogenous NO donors. It was previously demonstrated, however, that there is no significant difference in NO catalysis in the presence of GSNO from free CuBTTri particles versus CuBTTri immobilized in a chitosan matrix as determined by chemiluminescence (36). This suggests that NO-related anithrombogenic effects will be preserved when CuBTTri is incorporated into a polymer, pending the ability of the NO-donors to diffuse through the polymer matrix.

We hypothesized that CuBTTri would reduce MA specifically as the TEG MA has a strong linear relationship with platelet count and activity; and CuBTTri catalyzes NO which is a known platelet inhibitor (37). While this effect was assessed by TEG, we also investigated the impact of CuBTTri on platelets more directly using platelet aggregometry. Additionally, to demonstrate the catalytic activity of CuBTTri we added GSNO (50 µM) as a positive control to see if CuBTTri would enhance platelet inhibition in the presence of this endogenous NO-donor. In whole blood, GSNO alone had no effect on platelet aggregation which is likely due to the NO-scavenging effects of hemoglobin (Hb). Both cell-free and intraerythrocytic Hb have been shown to abolish the inhibitory effects of GSNO on collagen induced platelet aggregation in platelet rich plasma (38, 39). Interestingly, CuBTTri both with and without GSNO decreased platelet aggregation in whole blood. Accelerated NO release from GSNO in the presence of CuBTTri has been reported in real-time using chemiluminescence, where CuBTTri particles caused nearly a 65-fold increase in NO release relative to the thermal decomposition of GSNO alone (36). The ability of CuBTTri to accelerate NO release from GSNO could explain why platelet inhibition was observed in the CuBTTri+GSNO group, but not in the GSNO group. Others have reported that high concentrations
of NO-donors such as DEA/NO can inhibit platelets despite the presence of Hb if the heme ligands become saturated (40). By accelerating NO release through catalysis, CuBTTri may sufficiently increase NO levels so that NO is available to interact with platelets despite the scavenging effects of Hb. Alternatively, the fact that there was no difference in platelet aggregation between the CuBTTri and CuBTTri+GSNO groups could imply that GSNO-derived NO was less substantive or effective in platelet inhibition relative to NO catalyzed from other endogenous donors. It has been reported that the NO release profile of structurally distinct NO-donors varies from donor to donor, and that platelet inhibition can occur by independent mechanisms for different donors (40). For example, addition of a selective guanylate cyclase inhibitor, quinaxallin-1-one, limited but did not abolish inhibition of collagen stimulated platelet aggregation in PRP with GSNO and RIG200 (a novel S-nitrosothiol), but did not significantly reduce the inhibitory effect of DEA/NO. Presence of Hb reduced the inhibitory effects of GSNO, RIG200 and DEA/NO, but had a less pronounced effect in the DEA/NO group (40). Alternatively, the platelet inhibition observed may be due to another property of the CuBTTri that cannot be definitively determined in this study. We do not believe that the observed platelet inhibition is due to the particulate nature of the CuBTTri, as there was no change in platelet aggregation in the metal-based framework control group versus the Saline group, and CuBTTri substantially inhibited platelet aggregation relative to both groups.

We repeated the platelet aggregometry investigation in PRP to eliminate the scavenging effects of Hb. CuBTTri again inhibited aggregation relative to the controls; and there was no difference between the Saline and Zeolite groups, again suggesting that the presence of the inorganic-organic particulate species was not a significant contributor to the observed decrease in platelet aggregation. GSNO alone inhibited platelet aggregation, which is consistent with previous reports (40). The addition of CuBTTri to GSNO had an enhanced effect on platelet aggregation,
which as previously mentioned may be due to the demonstrated ability of CuBTTri to enhance NO release from GSNO (41). This effect may be amplified in the PRP due to the absence of an NO-scavenging agent, therefore eliminating any competitive interactions.

We observed an accelerated rate of fibrinolysis (elevated LY30 and LY60) in the high dose CuBTTri group, relative to the vehicle control group, which may be attributed to the role of NO in promoting dissolution of small platelet aggregates (42). NO derived from endogenous donor species has been shown to decrease surface expression of the platelet fibrinogen receptor GPIIb/IIIa, which is essential for sustained platelet aggregation (43); however, we did not observe platelet disaggregation during platelet aggregometry. We also observed numerically elevated LY30 and LY60 values in the Zeolite and Mil-100 Groups, which were not significantly different from the CuBTTri group. This suggests that the increase in LY30 and LY60 we observed is likely not specific to CuBTTri per se but rather is attributable effects of particulate organic-inorganic materials.

While this initial study has provided valuable information towards understanding fundamental effects of CuBTTri, a limitation is that it cannot be assumed that CuBTTri will have identical effects when incorporated into a polymer. Utilization of CuBTTri for medical applications will be dependent upon preservation of the anti-thrombogenic effects observed once the particles are incorporated into ECLS circuitry components. Once incorporated into medical polymers, the ability of NO-donors to diffuse into the polymer matrix, as well as the ability of NO-donors to interact with the catalyst under flow conditions will be key questions to address. Importantly, the scale of potential NO effects in-vivo cannot be conjectured from this report without targeted multi-day studies in large animals with human-like physiology. Such studies previously demonstrated the beneficial effects of partial (33) and full ECLS for reduction of
ventilator settings and as adjuncts to mechanical ventilation in severe ARDS (44) but were performed using systemic heparinization. This ex vivo experiment paves the way for incorporating CuBTTrri into ECLS circuitry. Pending future in vivo experiments utilizing CuBTTrri embedded polymers, this approach may reduce or eliminate anticoagulation requirements for ECLS and shift the current practice of anticoagulating the patient to an approach in which the circuit is constructed from antithrombogenic components. Future studies will require evaluation of blood exposure to CuBTTrri incorporated into biopolymers at physiologically relevant ECLS flow rates (0.5 L/min/m² – 5 L/min/m²). In vivo circulation studies will also provide further insight as to potential systemic effects of prolonged blood exposure to an NO catalyst during extracorporeal circulation; although the reversible nature of binding and short half-life of NO lend confidence that pronounced systemic effects are unlikely (45, 46).

In conclusion, ex-vivo exposure of CuBTTrri to blood led to reduced clot strength and platelet aggregation which provides preliminary evidence of the potential utility of this MOF as an antithrombogenic agent during ECLS. Pending further preclinical large animal testing this approach may revolutionize critical care eliminating the need for systemic anticoagulation of the patient by mitigating contact activation of the coagulation cascade in the circuit.

4.6 References.


Chapter 5:

Tethered-liquid omniphobic surface coating reduces surface thrombogenicity, delays clot formation and decreases clot strength ex vivo

5.1 Abstract.

5.1.1 Aims. Hemocompatible materials for extracorporeal life support (ECLS) technology are investigated to mitigate thrombotic complications associated with this therapy. A promising solution is an omniphobic bilayer coating, tethered liquid perfluorocarbon (TLP), which utilizes an immobilized tether to anchor a mobile, liquid surface lubricant that prevents adhesion of blood components to the substrate. In this study, we investigated the effects of TLP on real-time clot formation using thromboelastography (TEG). TLP was applied to TEG cups, utilizing perfluorodecalin or FluorLube63 as the liquid layer, and compared to uncoated cups. Human blood (n=10) was added to cups; and TEG parameters (R, K, α-angle, MA, LY30, LY60) and adherent thrombus weight were assessed.

5.1.2 Findings. TLP decreased clot amplification (α-angle), clot strength (MA) and adherent clot weight (p<0.0001). These effects were greater with FluorLube63 versus perfluorodecalin (α-angle p<0.0001; MA p=0.0019; clot weight p<0.0001)). Reaction time (R) was longer in TLP-coated cups versus control cups with liquid lubricant added (p=0.0377). Percent fibrinolysis (LY30 and LY60) was greater in TLP versus controls at LY30 (p<0.0001), and in FluoroLube63 versus controls at LY60 (p=0.0021).

5.1.3 Conclusions. TLP significantly altered clot formation, exerting antithrombogenic effects. This reduction in surface thrombogenicity supports TLP as a candidate for improved biocompatibility of ECLS materials, pending further validation with exposure to shear stress.

5.2 Introduction.

Since the earliest reported cases in the 1970s extracorporeal life support (ECLS) technology has made marked advances in survivability from severe trauma and renal and respiratory failure (1). ECLS circuitry consists of vascular access catheters, connective tubing, a treatment membrane and mechanical pump. Blood is pumped to and from the patient through connective tubing to the treatment membrane which supplements the function of the failing organ. The membrane off-loads the work of the native organ, allowing it to rest and recover. New applications, including bridge to transplantation, extended support for chronic lung failure, and personalized or “dynamic” renal replacement therapy are in early use or are under development (2, 3). As the scope of application of these devices is developed, one unresolved limitation continues to be debated: a solution to coagulation management.

Interaction of blood with plastic components of ECLS circuitry causes both hemostatic and hemorrhagic complications, which can coexist simultaneously in the same patient. Contact of blood with foreign surfaces in the circuitry leads to protein adsorption and fibrin deposition. Platelets and leukocytes become activated with exposure to fibrin deposits, causing release of soluble pro-inflammatory mediators and activation of complement factors (4, 5). Additionally, shear stress, turbulence and pressure drops generated by the circuit pump cause hemolysis, blood activation and introduction of procoagulant microbubbles into the circulation. To maintain circuit patency and prevent thrombus formation, anticoagulation is required (6).
Systemic anticoagulation with unfractionated heparin is the clinical standard for coagulation management of patients (6). Heparin, however, does not effectively address the coagulation disturbances that occur during ECLS, such as platelet activation and consumption, and protein adsorption (7). Additionally, systemic heparin alters native blood parameters causing untoward effects; and highly individualized patient responses significantly complicate care management (4, 6).

An alternative approach to coagulation management is to improve the biocompatibility of the circuit materials. This strategy directly addresses the coagulopathic impact of the foreign circuit surfaces, which would decrease the need for systemic anticoagulation. Such a material would not only improve patient care for ECLS, but also have applications for indwelling line placement and intravascular interventions. It would also extend ECLS therapy to patients in whom systemic anticoagulation is contraindicated, such as those with trauma or burns.

A promising solution is the omniphobic surface coating, tethered liquid perfluorocarbon (TLP). This coating was inspired by the *Nepenthes* pitcher plant, which has a roughened, tooth-like surface that holds in place a thin layer of liquid water. This super-hydrophilic surface inhibits adhesion of insects, causing them to slide down the pitcher rim and become trapped by the carnivorous plant (8). TLP is a bilayer coating consisting of the following components: 1) a tethered perfluorocarbon (TP): an immobilized perfluorocarbon layer that is covalently bound to an underlying substrate and acts as a tether; and 2) liquid perfluorocarbon (LP): a mobile, liquid surface layer that is retained on the substrate surface by the immobilized tether layer, imparting anti-adhesive properties to the substrate. This omniphobic liquid-infused coating repels both hydrophilic and hydrophobic materials, including clotting components of blood. It has been
demonstrated that TLP prevents the attachment of thrombus and pathogenic microbes to the surface of an array of medical grade materials, including plastics, glasses and metals (9).

The objective of this study was to investigate the effect of TLP on real-time clot formation in whole human blood exposed to TLP coated materials using thromboelastography (TEG). We compared two different LPs for the mobile surface layer: perfluorodecalin (PFD) and Fluorolube 63 (FL63). TEG is a viscoelastic clotting test that has been used to investigate the impact of various biomaterials at the blood/biomaterial interface in *ex vivo* blood preparations (10-13). It has been identified as a useful screening tool to select promising biomaterial candidates for *in vivo* testing (14). This method generates the following clinically relevant clot formation measures: reaction time (R), which is the time from start of test to first detection of clot formation (corresponding to 2 mm amplitude); initial clot formation time (K), which is the time from R to formation of a 20 mm amplitude clot; amplification rate (α-angle), which is related to the speed of fibrin formation and cross-linking; clot strength (MA), which is the maximum amplitude detected and is related to clot stability; and percent fibrinolysis at 30 min and 60 min after MA is detected (Ly30 and Ly60) (see Figure 5.1 for diagram of TEG values). We hypothesized that the TLP coating increases clot formation time and reduces clot strength by inhibiting surface adhesion of blood components.

### 5.3 Methods

**5.3.1 Preparation of TEG cups:** Standard clear TEG cups (Haemonetics Corp.; Braintree, MA) were exposed (45 s) to low-pressure (150-250 mTorr) radio frequency (13.56 MHz) oxygen plasma at 100 W. Cups were then immersed in liquid silane solution of 5% v/v tridecafluoro-1,1,2,2–tetrahydrooctyl trichlorosilane for 24 h, then rinsed and dried to covalently attach a perfluorinated layer. Before TEG analysis, a thin layer of liquid perfluorodecalin (PFD group) or liquid Fluorolube 63 (FL63 group) was applied to the cups using a plastic transfer pipette.
Figure 5.1 Diagram of thromboelastography parameters. Reaction Time (R) is the time from sample placement and start of test until detection of a 2mm amplitude clot. Initial Clot Formation Time (K) is the time from R until a specific clot strength is achieved (20 mm amplitude). Amplification rate (angle $\alpha$), measures the speed of fibrin formation and cross-linking. Clot Strength (MA) is the maximum amplitude of the tracing, representing strength and stability of the clot. LY30 is a measure of fibrinolysis and clot retraction, which is determined by the percent reduction in MA after 30 minutes. LY60 (not shown) is the percent reduction in MA after 60 minutes.
Uncoated standard TEG cups were used as a control (CTR group). As an additional control, liquid perfluorodecalin was applied to standard TEG cups without the perfluorcarbon tether layer (CTR+LP group) to account for volume changes.

5.3.2 Experimental procedures. Whole blood was collected in a de-identified fashion at the U.S. Army Institute of Surgical Research Blood Bank from healthy male and female donors between 18-40 years of age (n=10). Donors were non-smokers with no known coagulopathies. Blood was collected in 3.8% citrated BD Biosciences vacutainers (Franklin Lakes, NJ, USA) and analyzed within 2 hours of collection time at 37 °C. All coated and uncoated TEG cups were weighed before addition of blood, and after TEG was complete and non-adherent blood materials were removed by gentle inversion, to determine the adherent clot weight. 340 µL of citrated donor blood was added to each of the cups (CTR, CTR+LP, PFD, FL63) with 20 µL CaCl₂. Samples were run in duplicate for each treatment, and the average of the duplicates was taken for each donor.

5.3.3 Thromboelastography. Thromboelastography was performed without activating reagents using the Haemonetics Corporation TEG 5000 Hemostasis Analyzer System (Haemonetics Corp., Braintree, MA). The following variables of interest were recorded: reaction time (R), initial clot formation time (K), amplification rate (α-angle), clot strength (MA), and percent fibrinolysis at 30 min and 60 min after MA was detected (Ly30 and Ly60).

5.3.4 Statistical analysis. Statistical analysis was conducted using SAS 9.4 (Cary, North Carolina, USA). Tests were two-sided with an alpha = 0.05 for significance. The distribution of the data was tested using a Shapiro-Wilk test. The same donors were used in each group, so a mixed model with a random effect on donor was used to look for group differences. If a group
difference was significant, a Tukey adjustment was made for multiple comparisons. Results are expressed as mean ± standard deviation.

5.4 Results.

The reaction time, which is the time to start of clot formation (smallest detectable clot is 2 mm amplitude), was prolonged in the FL63 (9.77 ± 0.38 min) and PFD groups (9.79 ± 0.59 min) when compared to the CTR+LP group (8.42 ± 0.46 min); however, there was no statistical difference from the CTR group (9.07 ± 0.42 min) (Figure 5.2 A). The clot formation time, which is the time for the clot amplitude to increase from 2 mm to 20 mm, could not be determined for the FL63 group because the clots never reached 20 mm amplitude. In the PFD group, only 2 samples reached a 20 mm amplitude, with an average clot formation time of 10.1 ± 0.2 min. This was notably longer than the average clot formation time for CTR (2.5 ± 0.2 min) and CTR+LP (2.6 ± 0.5 min). The alpha angle, which represents the propagation phase of clot formation, was significantly reduced in the PFD (32 ± 1º) and FL63 groups (19 ± 3º) versus the controls (CTR = 56 ± 1º; CTR+LP = 51 ± 2º). Additionally, the alpha angle was significantly lower in the FL63 group versus PFD (Figure 5.2 B). The MA, which represents the clot strength, was significantly decreased in the FL63 group (9 ± 1 mm) versus the PFD group (15 ± 2 mm), and both were decreased versus the CTR (55 ± 2 mm) and CTR+LP (60 ± 1 mm) groups (Figure 5.2 C). LY30, which is the percent reduction in clot strength after 30 minutes, was greater in the FL63 group (27.7 ± 5.6 %) versus the CTR (3.9 ± 1.2 %) and CTR+LP groups (1.6 ± 0.6 %); it was also greater in the PFD group (11.2 ± 3.8 %) versus CTR+LP. LY60, which is the percent reduction in MA after 60 minutes, was greater in the FL63 group (40.4 ± 7.1 %) when compared to CTR (8.7 ± 1.8 %), CTR+LP (4.5 ± 0.9 %) and PFD (14.4 ± 4.7 %) (Figure 5.3 A and B).
Figure 5.2 Box plots depict (A) reaction time, (B) amplification rate and (C) clot strength determined by thromboelastography of human donor blood (n = 10 / group) using standard clear cups (CTR), standard clear cups with a thin liquid layer of perfluorodecalin (CTR + LP), tethered liquid perfluorocarbon (TLP) coated cup with a perfluorodecalin liquid layer (PFD), and TLP coated cup with a Fluorolube 63 liquid layer (FL63). The boundaries of the box indicate the 25th and 75th percentiles, with the median denoted within the box. The whiskers above and below the box indicate the 10th and 90th percentiles. Minimum and maximum values are shown (+). Statistical differences are noted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 5.3 Box plots depict the percent fibrinolysis at (A) 30 min (LY30) and (B) 60 min (LY60), as determined by thromboelastography of human donor blood (n = 10 / group) using standard clear cups (CTR), standard clear cups with a liquid layer of perfluorodecalin (CTR + LP), tethered liquid perfluorocarbon (TLP) cup with a perfluorodecalin liquid layer (PFD), and TLP coated cup with a Fluorolube 63 liquid layer (FL63). The boundaries of the box indicate the 25th and 75th percentiles, with the median denoted within the box. The whiskers above and below the box indicate the 10th and 90th percentiles. Minimum and maximum values are shown (+). Statistical differences are noted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
The adherent clot weight determined after the TEG run was significantly lower in the FL63 group (8.5 ± 2.4 mg) than PFD (95.7 ± 21.3 mg), and both were lower vs. the CTR (279.0 ± 7.0 mg) and CTR+LP groups (258.0 ± 46.0 mg) (Figure 5.4). Images of the TEG cups were taken after they had been inverted to demonstrate the reduction in adherent blood material in the TLP coated cups (Figure 5.5). The FL63 cups were nearly clean upon inspection at the end of the TEG run. Similar results were observed for the PFD cups, although adhesion to the pin cover was observed in approximately half of the PFD coated cups. Both the cup and pin cover portions of the CTR and CTR+LP cups were consistently covered in adherent blood material.

5.5 Discussion.

The principal findings of this study were: (1) TLP reduces clot formation and has anti-adhesive properties under the low shear conditions during TEG; (2) TLP reduces surface thrombogenicity in the TEG cup; and (3) the non-adhesive properties of the coating only occur when the LP is tethered to the material surface forming a bilayer. Additionally, using a quantitative analytical method we established that the FL63 LP performed better than the PFD in terms of decreasing blood adhesion and thrombus formation.

The reduced alpha angle in the TLP groups may be attributed to the anti-adhesive properties of the coating. Protein adsorption is a critical early step that mediates thrombus development on material surfaces. Specifically, fibrinogen is a key mediator of platelet adhesion to biomaterials under low shear stress (15). The surface properties of TLP inhibit fibrinogen adsorption, which prevents fibrin cross linking between the cup surface and pin. The reduction in MA is due to an inability of platelets to adhere or become activated when exposed to the TLP coating. Sun, et al investigated platelet adherence to nanostructured superhydrophobic films composed of poly(carbonate urethane)s with fluorinated alkyl side chains (16). They demonstrated
**Figure 5.4** Box plots depict the adherent clot weight of human whole blood (n = 10 / group) measured by weighing thromboelastography (TEG) cups before addition of blood and after TEG was complete and cups were gently inverted. Standard clear cups (CTR), standard clear cups with a liquid layer of perfluorodecalin (CTR + LP), tethered liquid perfluorocarbon (TLP) cup with a perfluorodecalin liquid layer (PFD), and TLP coated cup with a Fluorolube 63 liquid layer (FL63) were compared. The boundaries of the box indicate the 25th and 75th percentiles, with the median denoted within the box. The whiskers above and below the box indicate the 10th and 90th percentiles. Minimum and maximum values are shown (+). Statistical differences are noted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 5.5 Representative image of thromboelastography (TEG) cups after TEG was complete and cups were gently inverted to remove non-adherent blood. Standard clear cups (CTR), standard clear cups with a liquid layer of perfluorodecalin (CTR + LP), tethered liquid perfluorocarbon (TLP) cup with a perfluorodecalin liquid layer (PFD), and TLP coated cup with a Fluorolube 63 liquid layer (FL63) were compared.
that following exposure to platelet rich plasma these films had minimal platelet adhesion (fewer than 10 per sample), and observed platelets were in a rounded, inactive conformation. This was a stark contrast to control films where platelet adhesion estimated from SEM images was $7 \times 10^5$ cm$^2$ with the majority of platelets in a deformed/active conformation with pseudopods clearly observed.

Our results add to the evidence presented by Leslie, et al, demonstrating the reduced surface thrombogenicity of TLP (with PFD as the mobile, liquid layer) in a series of blood preparations (9). In this study, blood adhesion to material surfaces was quantified by determining the minimum angle required for a droplet of citrated blood to slide across the surface of the material. For TLP treated acrylic, the minimum angle required for the blood to slide was less than 1 degree, as compared to 90 degrees for the control uncoated materials. These results were maintained even after the materials had been exposed to fluid shear strain ($1,000 \text{ s}^{-1}$) for 16 hours. When heparinized blood (0.25 U/mL) was exposed to TLP-treated and untreated acrylic and polysulfone segments for 90 minutes, percent fibrin-covered area and platelet adhesion were significantly reduced with the TLP coating. Additionally, to investigate the performance of the tubing in an in vivo flow setting, TLP coated polyvinyl chloride (PVC) blood perfusion tubing was assembled into a femoral arteriovenous shunt for 8 hours. In unheparinized pigs, 100% of the TLP circuits maintained patency over the 8 hour period compared to only 20% of control circuits. There was also a 2.5-fold reduction in circuit occlusion, as determined by computerized image analysis of the lumens.

In our current study, the anti-adhesive effects observed in the TLP groups (both FL63 and PFD) did not occur in the CTR+LP group, indicating that the LP must be tethered to the substrate for these effects to occur. The liquid perfluorodecalin alone did not impart any reduction in clot
formation parameters or adherent material. The clean appearance of the TEG cups and reduction in adherent clot material in the TLP groups suggests that the bilayer remains intact with exposure to blood under low shear conditions during the >75 min TEG run. Immobilized PFD has been shown to remain in place under flow with minimal leaching of the LP layer, which is important for application on extracorporeal circuits. This was demonstrated with PFD applied to a fluorinated surface on the interior of PVC tubing (17). The tubing was incorporated into a closed circuit filled with deionized water driven by a peristaltic pump and exposed to flow rates of 10-90 mL/min for 16 hrs. Gravimetric and sliding angle analyses demonstrated that the tubing retained the PFD lubricant, and gas chromatography with electron capture detection of the water samples collected from the circuit showed minimal leaching of lubricant from the material surface. Further studies are needed to examine the stability of the coating with exposure to flow rates utilized for extracorporeal circulation (100 mL/min to 1 L/min for dialysis and partial lung support, up to 3-7 L/min for extracorporeal membrane oxygenation/full lung support).

The superior performance of FL63 LP versus PFD may be due to the lower volatility of FL63 compared with PFD. This increases ease of handling by reducing evaporation of the lubricant during the coating process. PFD is also less viscous (2.94 cSt at 25 °C) than FL63 (12.0 cSt at 25 °C), meaning less energy is required to move the PFD liquid along the surface with the same amount of shear force. This could result in a thinner layer of lubricant and potential lubricant stripping of PFD in comparison to FL63; however, PFD is a desirable candidate for medical applications due its clinical use and familiarity as a blood substitute (18).

An important limitation of this study is that the low-shear environment in the TEG cups does not account for the impact of shear-stress on blood-biopolymer interactions. This is an important consideration for application of TLP to biomaterials under flow, and future studies are
needed to investigate the performance of TLP coating under ECLS-relevant flow conditions. An additional limitation is that the measure of adherent clot weight did not include a characterization of the non-adherent blood that was removed from the cup during inversion. Further analysis is needed to assess the coagulability of the liquid blood removed from the TLP cups.

Development of hemocompatible surfaces for ECLS is vital, as systemic heparin administration does not effectively address surface mediated coagulation disturbances and complicates patient management. Heparin binds to antithrombin III (ATIII), inducing a conformational change that increases ATIII inhibition of thrombin and other clotting enzymes; however, heparin will not dissociate thrombin already bound to fibrin or material surfaces, and will not inactivate platelets (4). Additionally, the activity of heparin is altered by the presence of plasma proteins; and it is neutralized by high-molecular weight von Willebrand factor multimers and platelet factor 4 (PF4), both released by activated platelets (19). Cumulatively, this leads to an unpredictable drug response from patient to patient. Heparin induced thrombocytopenia (HIT) is an additional concern occurring in patients who develop antibodies against heparin-bound PF4. Other anticoagulants, such as direct thrombin inhibitors, are gaining favor in certain centers due to more predictable pharmacokinetics and ability to inhibit thrombin bound to fibrin clots (19); however, extensive use of these agents requires standardization of administration protocols and monitoring techniques, as well as improved understanding of reversal agents. There is currently no universal guideline for administration of anticoagulants or coagulation monitoring for ECLS patients, resulting in highly variable, center-specific hemostasis management protocols (20, 21).

Current generation ECLS devices utilize immobilized heparin surface coatings (e.g. Bioline®, Carmeda®) to decrease the risk of thrombus development and need for systemic anticoagulation; however, these coatings alone are not robust enough to prevent thrombus
formation, and supplementary use of systemic anticoagulants remains clinical practice (22, 23). This is in part due to the fact that heparin cannot eliminate platelet adhesion and aggregation on the circuit surface, which is a leading cause of circuit clotting (2). Additionally, studies caution against the use of these devices when HIT is suspected, as the HIT immune reaction has been observed to continue, even after withdrawal of systemic heparin, when a heparin-immobilized surface is used (24).

The anti-adhesive effects observed in this study, along with the previously reported data, identify TLP as promising candidate for surface coating of ECLS devices. Numerous studies have identified the clinical need for such a biocompatible coating to replace the use of heparin (25). As ECLS devices become more sophisticated and specialized for long-term support, coagulation management and improved material biocompatibility are hurdles that must be solved. This necessitates the study of materials in benchtop models such as TEG and translational animal studies for development to clinical trials. Understanding the interaction of TLP with blood at the material interface provides important insight for development of this coating for ECLS application.

5.6 References.


Chapter Six:

Heparin-free extracorporeal life support using tethered liquid perfluorocarbon: a feasibility and efficacy study

6.1 Abstract

6.1.1 Objective. Coagulation management is the leading challenge during extracorporeal life support (ECLS). A non-adhesive, slippery coating called tethered liquid perfluorocarbon (TLP) has been developed to prevent adhesion of blood and pathogens on medical devices and materials. In this study, we investigated the efficacy of TLP compared to immobilized-heparin during 6 hours in vivo circulation without systemic heparin. We hypothesized that TLP coating permits heparin-free circulation without untoward systemic effects, while reducing thrombus deposition on ECLS circuitry when compared to standard immobilized-heparin coating.

6.1.2 Design. Translational Animal Study.

6.1.3 Setting. Animal Intensive Care Unit Research Facility.

6.1.4 Subjects. Six anesthetized, spontaneously breathing swine.

6.1.5 Interventions. ECLS circuits were coated tip-to-tip with TLP antithrombogenic coating and compared to industry standard heparin-coated circuits. A 19-F dual-lumen catheter was inserted for veno-venous ECLS (1L / min blood flow rate). Subjects were monitored for 6 hours of circulation without systemic heparin anticoagulation therapy.

6.1.6 Measurements and main results. Key vital signs and respiratory parameters, gas
transfer across the ECLS membrane lung, coagulation panel and histology were evaluated to assess the safety and functionality of the TLP coating during ECLS without heparin. Scanning electron microscopy (SEM), elemental mapping and digital imaging were used to assess thrombus deposition on materials after use. There were no group differences in key vital signs, gas exchange efficiency, coagulation and histology. In both groups, ECLS enabled a decrease in minute volume and end-tidal CO₂, with concomitant increase in pH versus baseline (p<0.05). SEM and digital imaging revealed significant thrombus deposition on membrane lungs from control heparin-coated materials, which was reduced or absent on TLP-coated materials.

**6.1.7 Conclusions.** TLP coating permitted heparin-free circulation without altering ECLS device safety or function. TLP also prevented thrombus deposition in comparison with the industry standard immobilized-heparin coating. Pending multi-day *in vivo* testing, TLP could serve as a promising biomaterial solution to eliminate anticoagulation requirements during ECLS.

**6.2 Introduction.**

Coagulation management is the leading challenge during extracorporeal life support (ECLS). Despite advances in ECLS technology in the past decades that improve safety and simplicity, the issue of coagulation management remains unresolved (1, 2). Both bleeding and thrombotic complications limit the duration of use and range of applications of this life-saving therapy (3). All current forms of ECLS require systemic anticoagulation to prevent thrombotic complications; however, anticoagulation protocols are highly variable among centers with no accepted universal guidelines (4).

An alternative approach to systemic anticoagulation during ECLS is to improve the hemocompatibility of the device materials. A variety of strategies are under investigation, with the leading approach being immobilization of heparin on circuit surfaces (4). Other surface
modifications include hydrophilic materials that repel blood cells and proteins (5); immobilized albumin to competitively occupy sites for protein adsorption (6); zwitterionic surfaces containing phosphorylcholine-like residues (7, 8); and nitric oxide (NO) releasing materials that produce NO with similar flux as in the endothelium, inhibiting platelet activation (9, 10). While these approaches have been shown to improve surface hemocompatibility, an optimal solution to address both the protein adsorption and platelet activation that drive foreign surface-induced thrombosis has yet to be fully developed.

A novel approach to prevent thrombus formation on medical devices is a coating called tethered liquid perfluorocarbon (TLP) (11). This bilayer coating consists of a tether layer that is covalently bound to the material surface, and a non-adhesive liquid surface layer that prevents adhesion of blood and thrombus formation (Figure 6.1). The liquid lubricant is highly attracted to the immobilized tether, preventing wash-out of the lubricant into the blood as it passes over the surface. This coating has been applied to an array of medical polymers and shown to reduce adhesion and activation of blood components including fibrinogen and platelets ex vivo (11). Additionally, TLP coating reduced thrombus formation and maintained patency in an 8 h porcine arterio-venous shunt model (11). While this coating has demonstrated promising antithrombogenic effects during low-flow circulation (~10 – 20 L/hour), the coating has not been evaluated when applied to a standard ECLS circuit at clinically-relevant flow rates for extracorporeal CO₂ removal (ECCO₂R) (0.5 – 2 L/min).

The objective of this study was to evaluate the feasibility, efficacy and safety of TLP coating applied to ECLS circuits and catheters during ECCO₂R in spontaneously breathing anesthetized swine. We hypothesized that TLP-coating permits heparin-free ECLS and prevents
Figure 6.1 Schematic of extracorporeal circuit components to which tethered liquid perfluorocarbon (TLP) was applied. The components of the TLP bilayer are depicted. Specific segments of the extracorporeal circuit and catheter that were evaluated in this study are labelled.
thrombus formation compared to commercially produced heparin-coated circuits. Further, we hypothesized that TLP coating is safe and does not impede gas exchange efficiency. Using the industry standard 6 hour timeline for evaluation of biomaterials, we tested this hypothesis in swine during 6 hours of extracorporeal circulation without heparin, comparing TLP to immobilized heparin coating. We assessed vital signs, coagulation status, gas exchange efficiency, histology and thrombus deposition on circuit materials.

6.3 Materials and methods.

This study was carried out in compliance with the Animal Welfare Act, implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council. The Bridge PTS Animal Care and Use Committee approved all research conducted. The Bridge PTS facility is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC, Intl.).

6.3.1 ECLS equipment. An ECLS Circuit with permitted blood flow between 0.2 – 2.8 L/min was utilized in this study with Avalon Elite® 19 Fr bi-caval dual-lumen catheters (Maquet Getinge Group; Rastatt, Germany). A total of 3 TLP-coated and 3 uncoated devices were available for this feasibility study. In the TLP group, standard circuits (tubing, pump and membrane) and catheters as provided by manufacturer were coated tip-to-tip with TLP (FreeFlow Medical Devices, LLC; Lancatser, PA) (see Appendix D). ECLS Circuits with BIOLINE® immobilized-heparin coating from the manufacturer were used in the control (CTRL) group with standard catheters.

6.3.2 Animal preparation. Non-pregnant female Yorkshire swine (51 ± 8 kg) were endotracheally intubated and anesthetized using inhaled isoflurane (0.5 – 5% volume in oxygen). Animals were spontaneously breathing (F\textsubscript{R}O\textsubscript{2} = 100%) with tidal volume and minute ventilation measured hourly. Intravenous lines were placed for monitoring of vital signs and fluid
administration (see Appendix D). Continuous EKG, arterial blood pressure, oxyhemoglobin saturation (SpO₂), respiratory rate (RR) and end-tidal CO₂ (etCO₂) were monitored using the Dräger Infinity® M540 Monitor (Drägerwerk AG; Lübeck, Germany).

6.3.3 Initiation of ECLS. A 2000 U heparin bolus was administered to prevent thrombotic complications during cannulation. The 19 Fr dual-lumen catheter was percutaneously inserted into the right jugular vein for veno-venous (VV) ECLS. Blood flow through the circuit was 1 L/min, and the sweep gas (100% O₂) was 5 L/min. Following cannulation, no heparin was administered.

6.3.4 Measurements. All vital signs and monitoring tests were recorded following stabilization of the animal after instrumentation for baseline (BL), immediately post-initiation of ECLS (post-ECLS), and hourly for six hours. Coagulation measurements were taken at BL, post-ECLS, and at 3 and 6 hours post-ECLS.

Vital Signs included heart rate, mean arterial blood pressure (MAP), SpO₂, RR, tidal volume, minute ventilation and etCO₂. ECLS circuit arterial pressure (P₉₅), venous pressure (Pᵥₑₙ), internal pressure (Pᵢₙ), transmembrane pressure (∆p), and pump revolutions per minute (RPM) were recorded hourly. Blood gases (pH, partial pressure CO₂ (pCO₂) and O₂ (pO₂), oxygen saturation (SO₂) and lactate) were analyzed using the i-STAT1 Analyzer (Abott; Chicago, IL). Systemic blood samples were collected from the femoral artery, and pre-/post-membrane blood samples were collected from the circuit immediately before and after the membrane lung (ML) for determination of gas exchange efficiency (see Appendix D).

Coagulation testing included prothrombin time (PT), activated partial thromboplastin time (aPTT), activated clotting time (ACT), thromboelastography (TEG), antithrombin III (ATIII), von
Willebrand factor (vWF), fibrinogen, d-dimer, and plasma free hemoglobin (PFHb) as previously reported (see Appendix D) (12, 13).

6.3.5 Post-ECLS procedures. Following 6 hours ECLS, animals were deeply anesthetized and euthanized by exsanguination to prevent post-mortem clotting caused by euthanasia medications.

6.3.6 Histology. Tissue samples were taken from lungs, heart, liver, kidney, spleen and aorta for histology (see Appendix D). Images were evaluated by pathologist blinded to study conditions (14).

6.3.7 Evaluation of explanted ECLS circuits. After 6 hours, the ECLS circuit and catheter was explanted from the animal, rinsed with saline and fixed with glutaraldehyde for scanning electron microscopy (SEM) imaging (see Appendix D). The ML was disassembled to expose the polymethylpentene (PMP) gas exchange fibers. Samples were collected from the inlet face, center and outlet face of each ML for analysis as previously described (15). Samples from the dual-lumen catheter were collected from the inlet and outlet connections, the center of the catheter and tip (Figure 6.1). Tubing samples were collected from the pre-membrane and post-membrane lines.

6.3.8 Thrombus area. Digital images of the ML inlet, center and outlet PMP layers were used to determine the percent area of thrombus deposition on each layer, scored by 3 blinded reviewers using ImageJ Software (NIH; Bethesda, MD).

6.3.9 Field emission SEM imaging and EDS mapping. The ML, tubing and catheter were imaged using field emission SEM (FEI Teneo; Hillsboro, OR). Energy dispersive X-ray spectrometry (EDS) was used to determine the distribution and stability of the perfluorocarbon tether on the ML (see Appendix D).
6.3.10 Statistics. Statistics were performed using SAS 9.4 (Cary, NC). Tests were two-sided with alpha = 0.05 for significance. Groups were tested independently using one-way mixed models with repeated measures and Dunnett adjustment to assess changes from baseline. Between-group differences were examined using two-way mixed model with repeated measures and Tukey adjustment for multiple comparisons.

6.4 Results.

All animals were maintained on ECLS and survived to the end of study without heparin administration. There were no thrombotic or hemorrhagic events that would require early withdrawal of ECLS support, and no emergency procedures were required.

6.4.1 Vital signs and blood gases. There were no between group differences in vital signs or blood gases (Table 6.1). MAP was lower in the TLP group at 5 and 6 hours versus baseline. Other values were unremarkable except for a decrease in MV and etCO₂ and concomitant increase in pH in both groups vs. baseline (Table 6.1). Despite spontaneous breathing, ECLS enables reductions in minute ventilation in both CTRL (28-64% decrease) and TLP groups (20-52% decrease) (Table 6.1). Isoflurane rate (CTRL = 1.3 ± 0.3 %; TLP = 1.8 ± 0.2%) did not differ between groups.

6.4.2 ECLS gas exchange and circuit pressures. Trans-membrane reduction in pCO₂ ranged from 20-51% in CTRL and 25-41% in TLP (Figure 6.2). There was no difference in oxygen transfer (Figure 2). P_int was elevated in TLP versus CTRL at post-ECLS (Table 6.1).

6.4.3 Coagulation. There were no group differences in coagulation variables while on ECLS (Table 6.2). ACT and aPTT were numerically elevated in both groups post-ECLS due to pre-cannulation heparin bolus (Table 6.2). TEG clot strength was significantly higher in the CTRL
Table 6.1 Vital Signs and Circuit Parameters.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Baseline</th>
<th>PECLS</th>
<th>1 hr</th>
<th>2 hrs</th>
<th>3 hrs</th>
<th>4 hrs</th>
<th>5 hrs</th>
<th>6 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats per minute)</td>
<td>CTRL</td>
<td>117±29</td>
<td>114±25</td>
<td>107±25</td>
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<td>115±29</td>
<td>123±27</td>
<td>125±18</td>
<td>130±13</td>
</tr>
<tr>
<td></td>
<td>TLP</td>
<td>134±25</td>
<td>127±23</td>
<td>127±21</td>
<td>124±15</td>
<td>124±14</td>
<td>125±15</td>
<td>128±13</td>
<td>131±10</td>
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<td>Mean arterial pressure (mm Hg)</td>
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<td>76±3</td>
<td>70±4</td>
<td>72±1</td>
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<td>TLP</td>
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<td>74±3</td>
<td>73±6</td>
<td>64±2</td>
<td>64±4</td>
<td>60±4</td>
<td>59±1*</td>
<td>58±2*</td>
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<tr>
<td>Arterial pH</td>
<td>CTRL</td>
<td>7.28±0.04*</td>
<td>7.36±0.02*</td>
<td>7.51±0.07*</td>
<td>7.55±0.08*</td>
<td>7.54±0.06*</td>
<td>7.51±0.07*</td>
<td>7.53±0.07*</td>
<td>7.47±0.09*</td>
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<td>7.46±0.03*</td>
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<td>7.41±0.03*</td>
<td>7.39±0.05*</td>
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<tr>
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<td>423±40</td>
<td>477±17</td>
<td>473±18</td>
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<tr>
<td></td>
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<td>366±144</td>
<td>345±117</td>
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<td>47±10</td>
<td>45±8</td>
<td>51±11</td>
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<td></td>
<td>TLP</td>
<td>83±14</td>
<td>64±3</td>
<td>56±4</td>
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<td>53±1</td>
<td>54±3</td>
<td>59±2</td>
<td>61±7</td>
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<tr>
<td>Minute volume (L)</td>
<td>CTRL</td>
<td>6.1±0.8</td>
<td>2.9±1.5</td>
<td>2.2±1.3</td>
<td>2.8±1.6</td>
<td>3.0±2.0</td>
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<td>4.0±3.0</td>
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<td>6.7±1.0</td>
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<td>5.0±0.0*</td>
<td>4.0±1.0*</td>
<td>4.0±1.0*</td>
</tr>
<tr>
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<td>27±19</td>
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<tr>
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<td>TLP</td>
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<td>42±4*</td>
<td>27±11*</td>
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<td>24±10*</td>
<td>40±4*</td>
<td>40±4*</td>
<td>44±1</td>
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<tr>
<td>Internal membrane pressure (mmHg)</td>
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<td>--</td>
<td>137±4†</td>
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<tr>
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<td>TLP</td>
<td>--</td>
<td>174±9†</td>
<td>141±9</td>
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<td>142±7</td>
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<td>137±18</td>
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<td>Membrane inlet pressure (mmHg)</td>
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<td>-67±6</td>
<td>-66±4</td>
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<td>-77±12</td>
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<td>-76±10</td>
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<td>113±7</td>
<td>112±6</td>
<td>111±5</td>
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</tbody>
</table>

*Indicates within group change versus baseline; †Indicates between group difference.
Figure 6.2 Figure represents gas exchange efficiency of membrane lungs with tethered liquid perfluorocarbon (TLP) coating versus immobilized-heparin control coating (CTRL). Panel A lines represents mean ± standard error oxygen transfer (ml/min) measured from blood samples directly before and after passage through the membrane lung in the extracorporeal circuit. Panel B bars represent means ± standard error of pCO$_2$ for blood gases drawn from the pre-membrane (PRE) venous ECLS circuit line and post-membrane (POST) circuit line located immediately before and after blood passage through the membrane lung. Lines represent mean ± standard error of percent decrease in pCO$_2$ across the membrane. A two-sided test was performed with $p < 0.05$ accepted for significance. *Indicates within group change vs. baseline; †Indicates between group difference.
Table 6.2 Coagulation Data and Chemistry.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Baseline</th>
<th>Post-ECLS</th>
<th>3 hrs</th>
<th>6 hrs</th>
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<tr>
<td>Prothrombin time (sec)</td>
<td>CTRL</td>
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<td>13.3±0.1</td>
<td>13.1±0.2</td>
<td>13.1±0.1</td>
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<td>Activated partial thromboplastin time (sec)</td>
<td>CTRL</td>
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<td>70±8</td>
<td>22±5</td>
<td>22±5</td>
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<td>22±3</td>
<td>91±4</td>
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<td>20±3</td>
</tr>
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<td>Activated clotting time (sec)</td>
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<td>174±29</td>
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<td>89±1</td>
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<td>TLP</td>
<td>87±1</td>
<td>141±8</td>
<td>88±2</td>
<td>88±1</td>
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<td>Hemoglobin (g/dL)</td>
<td>CTRL</td>
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<td>9±1</td>
<td>9±1</td>
<td>9±1</td>
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<td>TLP</td>
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<tr>
<td>Hematocrit (%)</td>
<td>CTRL</td>
<td>33±4</td>
<td>31±2</td>
<td>28±1</td>
<td>29±1</td>
</tr>
<tr>
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<td>TLP</td>
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<td>30±2</td>
<td>26±2</td>
<td>28±2</td>
</tr>
<tr>
<td>Fibrinogen (mg/dL)</td>
<td>CTRL</td>
<td>200±27</td>
<td>169±14</td>
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<td>TLP</td>
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<td>198±34</td>
<td>156±11</td>
<td>157±8</td>
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<td>D-dimer (ug/mL)</td>
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<td>0.20±0.02</td>
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<td>CTRL</td>
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<td>91±1</td>
<td>90±2</td>
<td>86±5</td>
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<td>TLP</td>
<td>101±1</td>
<td>93±2</td>
<td>87±2</td>
<td>84±4</td>
</tr>
<tr>
<td>Von Willebrand Factor Antigen (%)</td>
<td>CTRL</td>
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<td>168±40</td>
<td>167±40</td>
<td>168±34</td>
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<td>TLP</td>
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<td>150±27</td>
<td>152±19</td>
<td>136±29</td>
</tr>
<tr>
<td>TEG Reaction Time (min)</td>
<td>CTRL</td>
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<td>5.9±0.5</td>
<td>5.5±0.7</td>
<td>5.2±0.5</td>
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<tr>
<td></td>
<td>TLP</td>
<td>6.5±0.4</td>
<td>6.1±0.5</td>
<td>5.6±0.4</td>
<td>5.8±0.5</td>
</tr>
<tr>
<td>TEG Clot Formation Time (min)</td>
<td>CTRL</td>
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<td>0.4±0.2</td>
<td>1.5±0.2</td>
<td>1.6±0.3</td>
</tr>
<tr>
<td></td>
<td>TLP</td>
<td>1.7±0.2</td>
<td>1.5±0.2</td>
<td>1.5±0.2</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>TEG Alpha Angle (degrees)</td>
<td>CTRL</td>
<td>64±9</td>
<td>64±5</td>
<td>68±2</td>
<td>63±3</td>
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<td>TLP</td>
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<td>64±6</td>
<td>67±3</td>
<td>60±2</td>
</tr>
<tr>
<td>TEG Clot Strength (mm)</td>
<td>CTRL</td>
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<td>71±1*</td>
<td>74±2*</td>
<td>75±1*</td>
</tr>
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<td>TLP</td>
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<td>70±1</td>
<td>72±1</td>
<td>72±1</td>
</tr>
<tr>
<td>TEG Lysis (%)</td>
<td>CTRL</td>
<td>0.8±0.3</td>
<td>1.9±0.5</td>
<td>1.4±0.2</td>
<td>1.3±0.5</td>
</tr>
<tr>
<td></td>
<td>TLP</td>
<td>1.3±0.6</td>
<td>1.0±0.7</td>
<td>0.8±0.4</td>
<td>0.6±0.5</td>
</tr>
</tbody>
</table>

*Indicates within group change versus baseline; †Indicates between group difference.
group versus TLP at baseline and was reduced within the CTRL group at all timepoints post-ECLS (Table 6.2).

6.4.4 Histology. Both groups showed atelectasis in dependent lung areas. There was no histopathologic evidence to suggest toxicity in either group. No evidence of thrombi or microhemorrhage was found.

6.4.5 Thrombus area. The percent area of thrombus deposition on the ML did not differ statistically between the groups but was numerically higher in CTRLs at the inlet (2x greater), center, and outlet. In both groups area of thrombus deposition decreased with progression from the inlet face (CTRL = 4.6 ± 1.0 %, TLP = 2.0 ± 1.1 %) to the center (CTRL = 0.7 ± 0.2 %, TLP = 0.3 ± 0.2 %) and the outlet (CTRL = 0.8 ± 0.7 %, TLP = 0.1 ± 0.0 %).

6.4.6 SEM imaging and EDS mapping. SEM imaging showed significant clot deposition on CTRL membranes, which was reduced or absent in TLP (Figure 6.3). Thrombus deposition was minimal on catheter and tubing samples in both groups (Appendix D). Fluorine was detected by EDS on all TLP membrane segments. The weight percent fluorine detected did not differ between the inlet (20.01 ± 3.26 wt. %), center (22.54 ± 3.74 wt. %) and outlet (27.82 ± 4.1 wt. %).

6.5 Discussion.

We compared TLP-coating to immobilized-heparin during 6 hours heparin-free ECLS in swine. Use of TLP and ECLS in this study was investigational and is currently not approved by the FDA. Our primary finding is that TLP permits heparin-free ECLS for 6 hours, and does not alter key vital signs, coagulation parameters, or gas exchange efficiency of the ML versus the clinical standard – immobilized-heparin. Histological evidence suggests that the coating is not toxic with 6 hours exposure. Based on SEM imaging, TLP prevents thrombus formation to a greater extent than immobilized-heparin. These findings have important clinical implications as
Figure 6.3 Field emission scanning electron microscopy images from membrane lungs with tethered liquid perfluorocarbon (TLP) coating versus immobilized-heparin control coating (CTRL). Panel A representative images from the inlet face of CTRL membrane lungs with thrombus deposition, platelet activation and adhesion and fibrin network formation; Panel B representative images from inlet face of TLP-coated membrane lungs with minimal cellular deposition and clean appearance. Panel C (CTRL) and Panel D (TLP) demonstrate the clean appearance of membrane lungs sections collected at the center (left image) and outlet (right image) face of the membrane.
coagulation management and bleeding are significant complications during ECLS, and TLP may be a biocompatible alternative to prevent clotting without heparin.

Perfluorocarbons have been utilized in many medical applications due to their multifaceted properties including hydrophobicity, oxygen solubility and biological inertness (16). Liquid perfluorocarbons have been used as oxygen carriers, blood substitutes (17), diagnostic reagents (18), and are under investigation for gene and drug delivery (16). This study describes a novel application for perfluorocarbons as a hemocompatible coating for ECLS.

A suitable hemocompatible coating for ECLS cannot impede gas exchange efficiency of the ML. We did not observe differences in CO\textsubscript{2} removal between groups. Interestingly, reduction in systemic CO\textsubscript{2} led to decreased respiratory drive and intermittent apnea in 2 TLP animals and 2 CTRLs. The PaCO\textsubscript{2} and pH shift we observed post-ECLS is similar to values reported by Batchinsky \textit{et al.}, in mechanically ventilated swine on low-flow ECLS system in whom, despite 50\% reduction in ventilator settings, normocarbia and normal pH were observed (19). Similar findings were reported by Karagiannidis \textit{et al.} using the ECLS system we utilized in this study (20), putting our results in context with reported results and suggesting that TLP does not affect CO\textsubscript{2} removal. O\textsubscript{2} transfer also did not differ between groups and was reasonable in comparison with reports for membranes with similar dimensions and flow rates (20, 21). This information is lacking from most studies investigating hemocompatible materials for ECLS, as the coating is generally applied only to tubing and catheters.

\(P_{int}\) was elevated in TLP post-ECLS, which was reduced by 1-hour (Table 6.2). This suggest that excess lubricant is stripped from the membrane surfaces at start of circulation. If excess lubricant was stripped from the ECLS surfaces, the volume was below that of intravenous delivery of perfluorocarbons administered clinically (22). For example, intravenous administration
of perflubron emulsion had no impact on bleeding time, (1) and function (23); and did not impact circulating immunoglobins, cytokines or complement activation (24). This is consistent with our findings, as no signs of systemic damage were observed.

We did not observe group differences in any coagulation measures during ECLS, demonstrating that hemocompatibility of the TLP is comparable to the current clinical standard (Table 6.2). There were no deviations from baseline in either group except for clot strength (TEG MA) in CTRLs. Clot strength, which has a linear relationship with platelet count, was reduced in CTRLs but was preserved in TLP (25). PFHb was lower than levels indicative of hemolysis, defined as > 50 mg/dL (4). Additionally, PFHb was comparable to reported results in swine on ECLS with continuous heparinization over 72 hours (19) and sheep on VV-ECMO without heparin for 5 hours (12). This lends support to the use of initially heparin-free ECLS in trauma patients with severe bleeding, in whom heparin-free protocols have been utilized until bleeding is resolved (26-28).

Artificial surfaces cause thrombosis through protein adsorption, platelet, leukocyte and red blood cell adhesion, thrombin generation and complement activation. This is presented on artificial surfaces mainly in the form of fibrin networks and platelet aggregates (29). Thrombus deposition on CTRL membranes, primarily on the inlet face, is consistent with our previously reported SEM evaluation of heparin-coated MLs after 14 hours circulation in sheep (15), suggesting that immobilized-heparin does not prevent thrombus formation on the ML. While some activated platelets were observed on the TLP membrane inlet, the vast interconnected network of fibrin was not observed. This inhibition of fibrin network formation prevents circuit occlusion and limits formation of thrombi that can impede gas exchange by increasing the diffusion barrier. Lehle et al. used SEM to analyze MLs from patients with severe acute respiratory distress syndrome and
noted pseudomembrane layers of blood deposits of 30-45 µm (wall thickness of PMP fibers was 75 µm), significantly increasing the gas diffusion barrier (30). Deposition of platelets and cellular debris on catheters occurred preferentially at the connection points (inlet and outlet) and tip (Appendix D). The varied diameter of the flow path at the connections can induce turbulence and promote surface contact. TLP catheters had fewer adherent activated platelets compared to CTRLs. Tubing had minimal deposition and looked like samples collected before use (Appendix D).

A limitation of this pilot study is the small sample size, due to availability of ECLS circuits; however, we believe the analysis we performed utilizing manufacturer’s complete circuits in animals with human-like cardiopulmonary physiology is extremely valuable to duplicate the flow conditions and shear stress that occur during ECLS. This provides a robust evaluation of the biomaterials in contrast to assessment of isolated circuit components. The use of healthy animals does not address coagulopathic complications observed in injured patients; however, in this initial study we removed this variable as underlying coagulopathy is highly individualized. The circulation time was selected to compare our results to the literature, as most evaluations of biomaterials for the Food and Drug Administration are conducted for 4-6 hours. We cannot assume the results observed will be preserved outside the time frame tested, and additional multi-day studies will be necessary to establish if TLP is a robust solution for heparin-free ECLS.

6.6 Conclusions.

We demonstrated safety, feasibility and efficacy of heparin-free ECLS for 6 hours using TLP-coating. We found that TLP prevents thrombus deposition more so than standard immobilized-heparin as observed by SEM. Finally, we did not observe a reduction in gas exchange performance of the ML caused by the TLP-coating, or any negative systemic effects. Future studies
are needed to evaluate TLP applied to ECLS circuits at a clinically relevant multi-day timeframe in models of lung injury that mimic human pathophysiology.

6.7 References.


Chapter Seven:

Evaluation of tethered-liquid perfluorocarbon coating for heparin-free extracorporeal life support: a 72-hour intensive care unit study

7.1 Abstract.

Extracorporeal life support (ECLS) is a class of technologies to support or replace the function of failing organs, such as the heart, lungs, kidney and liver. This technology has been utilized in diverse patient populations from neonates with respiratory failure to combat casualties with traumatic lung injury. The key limitation that prevents extended use of this life-saving therapy is the coagulation disturbances that occur when blood contacts the foreign surfaces of the devices, as well as the bleeding complications that result from the need to administer anticoagulant drugs to prevent circuit thrombosis. To address this challenge, biocompatible surface coatings are being developed to mitigate thrombosis and bleeding caused by shear stress and contact with the circuit materials, reducing or eliminating the need for anticoagulants.

Tethered-liquid perfluorocarbon (TLP) is a slippery, non-adhesive coating shown to improve the biocompatibility of medical polymers by preventing adhesion of plasma proteins and deposition of cellular debris on ECLS components. Here we examine a novel use of TLP applied to ECLS membranes, tubing, pumps and catheters during 72 hours of extracorporeal circulation in healthy swine (n=5/group) without use of systemic anticoagulation. We compare this heparin-free approach to the standard of care – heparin-coated ECLS circuit with
continuous heparin infusion – and evaluate coagulation response, instance of coagulopathic complications, gas exchange efficiency of the membrane lung and systemic effects. We hypothesized the TLP would prevent thrombotic circuit occlusion while preserving baseline platelet counts and coagulation values; without impeding the gas exchange performance of the membrane lung or causing untoward systemic effects. We found that while instance of bleeding complications was greater in controls (80%) versus TLP (20%), thrombotic circuit occlusions occurred in both groups (control = 20%, TLP = 40%), suggesting neither approach is sufficient for prevention of hemostatic complications during ECLS. Thrombus deposition and protein adhesion were similar on explanted membranes, and TLP did not preserve platelet or blood cell counts relative to controls. TLP required higher ECLS sweep gas flow rates and blood flow, suggesting gas exchange performance was impeded. As we evaluated the impact of ECLS on coagulation in healthy subjects we concluded that neither TLP nor the standard care is a safe or efficacious solution to prevent coagulopathy induced by extracorporeal circulation and blood-biomaterial contact. Further development of a multi-functional coating that regulates hemostasis through complementary mechanisms as occurs in the endothelium may be the ideal solution. Such an approach could incorporate TLP with other agents that inhibit thrombin or cause platelet quiescence, for example.

7.2 Introduction.

Thrombosis, coagulopathy and inflammation are major issues associated with the use of blood-contacting medical devices. This challenge is apparent during ECLS where exposure of blood to foreign materials, shear stress from the blood pump, and turbulent flow caused by circuit and catheter connections causes significant damage to blood cells. When blood contacts the circuit, protein adsorption and contact pathway activation rapidly occur followed by platelet
activation, adhesion and thrombus formation (1, 2). Thrombi in the circuit can impede flow and reduce the performance of the membrane – such as inhibiting gas diffusion across the membrane lung (3). Simultaneously, progressive platelet consumption, activation of clotting factors and hemolysis cause risk of consumptive coagulopathy and bleeding (4, 5). Additionally, circulating blood cells become activated when exposed to developing thrombi and adsorbed proteins, as well as turbulent flow, causing release of pro-inflammatory factors and complement activation as the blood is returned to systemic circulation (6, 7).

The risk of thrombotic complications during ECLS necessitates systemic anticoagulation therapy; however, there are currently no universal guidelines for anticoagulant administration during ECLS, resulting in highly variable, center-specific protocols (5, 8). The task of maintaining therapeutic anticoagulation amidst circuit-induced coagulation disturbances, underlying conditions and frequent transfusions is extremely challenging. The result is frequent bleeding complications, which can occur at the cannulation site, concomitant wounds, in the airway, gastrointestinal tract, and can even cause lethal cerebral hemorrhage (9, 10).

An alternative to systemic anticoagulation is to prevent foreign surface-induced thrombosis and maintain circuit patency using bio-compatible surface coatings. This approach provides localized anticoagulation at the blood-biomaterial interface. Modern ECLS devices utilize biocompatible coatings such as immobilized heparin and surface passivation with albumin; however, these coatings alone are insufficient to prevent coagulation disturbances, and thus systemic anticoagulation remains standard practice (11, 12). Other investigational surface coatings include nitric oxide (NO) releasing surfaces that produce a similar NO flux as occurs in the endothelium to provide localized platelet inhibition (13-15); immobilization of direct thrombin inhibitors to prevent thrombus formation with more direct pharmacodynamics than heparin (16);
and endothelialization of the circuit to cover the foreign-surfaces (17). While these approaches have shown promise in the laboratory setting, they have been primarily tested when applied to connective tubing segments only, or to segments of deconstructed membranes and catheters. Additionally, these materials are often evaluated using stationary incubation in blood; or in vivo under flow using an arterio-venous shunt, which does not account for the effects of the blood pump or adequately simulate the range of flows for cardiopulmonary (2-7 L / min) or partial respiratory support (500 mL – 2 L / min). While such studies are integral for evaluation and optimization of these materials, to fully understand the efficacy, longevity and functionality of these coatings evaluation using multi-day studies that closely simulate the clinical care scenario is necessary.

A promising biomaterial solution for ECLS is an omniphobic surface coating called TLP (18). TLP was developed to prevent adhesion of plasma proteins, cells, bacteria or any biological medium to medical polymer substrates. This coating was inspired by the Nepenthes pitcher plant, which has a roughened, tooth-like surface that holds in place a thin layer of liquid water. This super-hydrophilic surface inhibits adhesion of insects, causing them to slide down the pitcher rim and become trapped by the carnivorous plant (19). This bilayer coating consisting of the following components: 1) a tethered perfluorocarbon: an immobilized perfluorinated layer that is covalently bound to an underlying substrate and acts as a tether; and 2) liquid perfluorocarbon: a mobile, liquid surface layer that is retained on the substrate surface by the immobilized tether layer, imparting anti-adhesive properties to the substrate. This omniphobic liquid-infused coating repels both hydrophilic and hydrophobic materials, including clotting components of blood. For example, Leslie, et al, demonstrated that TLP prevents the attachment of thrombus and pathogenic microbes to the surface of an array of medical grade materials, including plastics, glasses and metals (18). Blood sliding angle on TLP- treated acrylic was less than one degree versus 90 degrees on control
materials; and results were maintained following exposure to fluid sheer strain (1,000 s-1) for 16 hours. When heparinized blood (0.25 U/mL) was exposed to TLP-treated and untreated acrylic and polysulfone segments for 90 minutes, percent of fibrin-covered area and platelet adhesion were significantly reduced with the TLP coating. Additionally, to investigate the performance of the tubing in an in vivo flow setting, TLP coated polyvinyl chloride (PVC) blood perfusion tubing was assembled into a femoral arteriovenous shunt for 8 hours. In un-heparinized pigs, 100% of the TLP circuits maintained patency over the 8 hour period compared to only 20% of control circuits. While these results are promising, this coating has yet to be evaluated when applied to all components of the ECLS circuit (coated “tip-to-tip” on all surfaces) and in multi-day studies that simulate clinical care conditions (18).

In this study we evaluated the use of TLP applied tip-to-tip to a low-flow (< 2 L/min blood flow rate) ECLS device for partial respiratory support (includes membrane, blood pump, connective tubing and catheter). ECLS is used as an adjunct therapy to mechanical ventilation during severe respiratory distress, as the artificial membrane lung supplements the work of gas exchange of the native lung, permitting reduction in ventilator settings and preventing ventilator-induced lung injury (VILI) (20, 21). Use of ECLS to mitigate VILI during severe respiratory distress has been used with good success since the H1N1 pandemic (22, 23).

We compared the TLP coated devices used without supplemental anticoagulation to the standard-of-care -- heparin-coated circuits with continuous heparin infusion -- for 72 hours of intensive care unit (ICU) care using ELCS in healthy, mechanically ventilated swine. We assessed coagulation, thrombus deposition, gas-exchange efficiency, efficacy and safety of the membrane lungs, systemic response and end-organ damage (Figure 7.1). The objective was to
Figure 7.1 Schematic of study design for evaluation of tethered-liquid perfluorocarbon (TLP) coating during 72 hours extracorporeal circulation in healthy swine. A) Depiction of TLP bilayer coating components including immobilized tether layer and mobile liquid surface layer. B) Timeline of key data collection points is shown. C) Key outcome measures to assess efficacy and biocompatibility of the TLP surface coating includes coagulation profile, thrombus deposition on circuit components, gas-exchange performance of the membrane lung (addition of oxygen (O$_2$) and removal of carbon dioxide (CO$_2$)), and systemic response including vitals and histology.
evaluate if TLP is safe and efficacious permitting heparin-free ECLS over 72 hours. We hypothesized that TLP maintains circuit patency and reduces thrombotic and bleeding complications relative to the standard-of-care; all while not altering gas exchange performance of the membrane lung or causing untoward systemic effects.

7.3 Methods.

This study was carried out in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council. An Institutional Animal Care and Use Committee (Bridge PTS, San Antonio, Texas) approved all research conducted in this study (Protocol BRIDGE PTS-17-08). Bridge PTS is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC, Intl.).

7.3.1 Equipment. A pediatric size ECLS Circuit compatible with the CARDIOHELP System (Maquet Getinge Group; Rastatt, Germany) with blood flow between 0.2 – 2.8 L/min was utilized in this study. Circuits consisted of a ROTASSIST 2.8 centrifugal blood pump with integrated pressure gauge, a membrane lung (0.98 m² surface area) with integrated pressure sensor, and ¼” tube connections. Avalon Elite® 19 Fr bi-caval dual-lumen catheters (Maquet Getinge Group; Rastatt, Germany) were used for veno-venous ECLS. A total of 5 TLP-coated and 5 uncoated devices were available for this study. In the TLP group, ECLS circuits (tubing, pump and membrane) and catheters as provided by manufacturer were coated tip-to-tip with the tethered layer of the TLP coating (FreeFlow Medical Devices, LLC; Lancaster, PA, USA) (see section 7.3.2). ECLS Circuits with BIOLINE® coating (immobilized albumin with covalently bound high molecular weight heparin) from the manufacturer were used in the control (CTRL) group with standard Avalon Elite® catheters (Maquet Getinge Group; Rastatt, Germany).
7.3.2 TLP coating. Application of TLP to ECLS circuits and catheters was carried out in 2 phases. First, we sent entire ECLS circuits and components to Free Flow Medical Devices, LLC (Lancaster, PA, USA) for attachment of the perfluorinated silane tether layer. The tethered circuits were returned to our lab for conductance of experiments. Application of the liquid perfluorocarbon lubricant layer was carried out by us immediately before priming of the ECLS circuit and cannulation. Namely, 150 µL of liquid lubricant, FluoroLube63 (FL63; Sigma-Aldrich; St. Louis, MO), was sterilized using a 0.2 µM filter and injected into the pre-membrane/venous face of the membrane lung. Once the membrane was saturated, FL63 was drained from the oxygenator into the pump and tubing components. The excess FL63 was then withdrawn, with ~30mL lubricant remaining in the circuit. The Avalon catheters with tethered layer applied were filled with FL63, then drained of lubricant immediately prior to cannulation.

7.3.2 Instrumentation. Female Yorkshire swine (CTRL = 48.6 ± 4.6 kg; TLP = 45.3 ± 2.6 kg; p > 0.05) were anesthetized with 6 mg/kg Telazol® (tiletamine/zolazepam) delivered intramuscularly (IM). Atropine sulphate (0.05 mg/kg) was administered IM to prevent airway secretions. Endotracheal intubation was performed via direct laryngoscopy using a 10 mm endotracheal tube. A surgical plane of anesthesia was achieved using inhaled isoflurane (0.5 – 5% volume in oxygen). A 16 Fr Foley catheter was inserted trans-urethrally. Urine output was measured by the BARD® CRITICORE® Monitoring System (BARD Medical; Covington, Georgia, USA) to titrate fluid infusion during multi-day ICU care. The right jugular vein, left carotid artery and the left and right femoral arteries and veins were cannulated with 7-8.5 Fr sheath introducers (Arrow International Inc.; Reading, PA, USA) placed percutaneously with ultrasound guidance. Arterial sheaths were cannulated with IV lines for monitoring arterial blood pressure (ABP) and for collection of systemic blood samples. Venous sheaths were cannulated with IV lines
for administration of fluids (normal saline) and anesthesia medications using an Alaris™ MedSystem III® Multi-Channel Infusion Pump (Alaris Medical Systems, Inc.; San Diego, CA, USA). Following venous line placement, animals were transitioned from inhaled anesthesia to total IV anesthesia (TIVA) for the remainder of the study. TIVA consisted of continuous infusion of fentanyl (1-4 mcg/kg/hr), ketamine (2-10 mg/kg/hr), midazolam (1-5 mg/kg/hr) and propofol (10-25 mcg/kg/min). Additionally, propofol boluses (1-3 mL) were delivered if evidence of spontaneous breathing, purposeful movement or discomfort were observed. Endotracheal intubation was converted to surgical tracheostomy using a 10.0 mm tracheostomy tube. Animals were volume-control ventilated (V500, Dräger Medical; Lübeck, Germany) with room air, initial tidal volume (V_T) of 10 mL/kg and respiratory rate (RR) of 10-14 breaths/min. Positive end expiratory pressure (PEEP) was 5 cm H_2O. Continuous EKG, arterial blood pressure (ABP) and oxyhemoglobin saturation (SpO_2) were monitored using the Dräger Infinity® M540 Monitor (Drägerwek AG; Lübeck, Germany). Animals were stabilized for 30 min prior to record of vital signs and collection of baseline samples.

7.3.3 Initiation of ECLS. Prior to cannulation, all ECLS circuits were primed according to manufacturer’s instructions using 1 L normal saline to which 5000 U of unfractionated heparin had been added. A 2000 U unfractionated heparin bolus was administered to prevent thrombosis during cannulation. A 19 Fr dual-lumen catheter was percutaneously inserted into the right jugular vein for veno-venous (VV) ECLS. The catheter was connected to the ECLS system in an air-free, wet-to-wet fashion. Blood flow through the circuit was set to 1 L/min, and the sweep gas (100% O_2) flow rate was set to 5 L/min. Following cannulation, heparin infusion was initiated in the CTRL group (40 U/kg/hr) and titrated to maintain an ACT of 50% above baseline value. No heparin was administered in the TLP group for the remainder of the study. Following collection
of data and blood samples immediately after initiation of ECLS (Post-ECLS time point), the ventilator settings were incrementally adjusted. First, $V_T$ was reduced by 2 cc/kg to achieve a peak airway pressure below 35 cm H$_2$O; then, $V_T$ was lowered to a minimum of 4 cc/kg. This progressive reduction of ventilator settings has been shown to reduce markers of VILI and pulmonary inflammation relative to the standard lung-protective ventilation protocol of 6 mL/kg $V_T$ in acute respiratory distress (ARDS) patients (24). RR was incrementally reduced to a target of 4 breaths / min; and FiO$_2$ and ECLS settings were titrated to achieve a P$_{a}$O$_2$ > 60 mmHg and pH > 7.2.

7.3.4 ECLS. Data were collected for 72 hours of extracorporeal circulation unless the circuit became occluded inhibiting flow (RPMs maximized at 5000, with $\Delta p > 200$ mmHg and decline in flow below 0.5 L / min), at which point the study was terminated. All vital signs, point-of-care monitoring tests and blood labs were taken at baseline (BL), immediately post-initiation of ECLS (Post-ECLS), and at 3, 6, 12, 24 48 and 72 hours post-ECLS (see Figure 7.1). Animals were continuously monitored by technicians, study staff and a physician throughout the experiment. If ECLS settings were insufficient to support the clinical condition of the animal (P$_{a}$CO$_2$ 35-45 mmHg; P$_{a}$O$_2$ < 60 mmHg), sweep gas flow rate and blood flow rate were increased accordingly. These changes were recorded and time to onset of required adjustments was reported. If required, vasopressors (epinephrine) were administered for blood pressure support and a loop diuretic (Furosemide, 0.5 mg) was given to increase urine output (if < 0.5 ml/kg/hour) and/or the risk of kidney injury was suspected. ECLS circuit parameters including arterial pressure (P$_{a}$rt), venous pressure (P$_{ven}$), internal membrane pressure (P$_{int}$), pressure drop across the membrane ($\Delta p$), and pump revolutions per minute (RPM) were recorded. Any hemorrhagic or thrombotic complications observed during circulation were recorded.
7.3.4.1 Blood labs and coagulation. To assess the impact of extracorporeal circulation on circulating blood and hemostasis, complete blood count was performed using the ADVIA 2120 hematology analyzer with veterinary software package (Siemens AG, Germany). Samples were collected in EDTA tubes and analyzed using porcine settings. Coagulation testing included prothrombin time (PT), activated partial thromboplastin time (aPTT), antithrombin III (ATIII), von Willebrand factor (vWF), and concentrations of fibrinogen and d-dimer measured in previously frozen (-80 ºC) platelet poor plasma (PPP) using the Stago STA Compact Max Analyzer (Stago; Parsippany, NJ, USA) as previously reported (25, 26). The PPP was prepared by dual centrifugation (3,000 g, 10 min, 4ºC) of citrated whole blood. Activated Clotting Time (ACT) was measured using the Hemochron Signature Elite Whole-Blood Microcoagulation System (Accriva Diagnostics; San Diego, CA). Thromboelastography (TEG) was performed using the TEG 5000 Hemostasis Analyzer System (Haemonetics Corp., Braintree, MA) according to manufacturer’s instructions. 3.8% citrated arterial whole blood was analyzed using the citrated kaolin assay and heparinase cups to neutralize the effects of heparin. The following variables of interest were recorded: reaction time (R), initial clot formation time (K), clot strength (MA), and lysis (Ly60). Platelet aggregation was measured by whole-blood impedance aggregometer (Multiplate® Analyzer, Roche Diagnostics; Basel, Switzerland) using ADP (final concentration 10.75 uM; ADPtest, Roche Diagnostics; Basel, Switzerland) and collagen (final concentration 3.2 ug/mL; Helena Laboratories; Beaumont, Texas) as activators; area under the curve was calculated as previously described (25). Plasma free hemoglobin (PFHb) was measured in EDTA plasma (dual centrifugation 3,000 g, 10 min, 4 ºC) by direct spectrophotometry as previously described (27). To assess systemic inflammatory
response and endothelial damage, EDTA plasma samples were frozen (-80 ºC) for later analysis of cytokines (IL-1b, IL-6, IL-8, IL-10 and TNF-α) using a MILLIPLEX Porcine Magnetic Bead Panel (Millipore Sigma; Burlington, MA) and Luminex® 200 Analyzer (Luminex Corporation; Austin, TX). Serum samples were collected in BD Vacutainer® serum collection tubes (Becton Dickinson; Franklin Lakes, NJ), incubated for 1.5 hours, centrifuged (3,000 g, 10 min, 4 ºC) and frozen for later analysis of syndecan-1 concentration using an ELISA kit (SEB966PO, Cloud-Clone Corp.; Katy, TX).

7.3.4.2 Vital signs, respiration and chemistry. Vital Signs and respiratory measures included heart rate, mean arterial blood pressure (MAP), SpO₂, RR, CO₂ production (V̇CO₂), minute ventilation (MV), plateau pressure (Pplat) compliance (Cdyn), pulmonary resistance and etCO₂. Urine output (UO), fluid balance, anesthesia rates and rectal core temperature were also recorded. Blood gases (pH, partial pressure CO₂ (pCO₂) and O₂ (pO₂), oxygen saturation (SO₂) and lactate) were analyzed using the i-STAT1 Blood Analyzer (Abott; Chicago, IL). Systemic blood gas samples were collected from the femoral artery; pre-membrane blood samples were collected from the right femoral venous line which was positioned to withdraw blood before entry into the ECLS catheter; and post-membrane samples were collected from a port located within the ECLS connective tubing directly after blood passage out of the membrane lung for determination of gas exchange efficiency. The membrane CO₂ removal was determined by the percent decrease in pCO₂ from a pre-membrane to post-membrane blood gas at each time point. The O₂ transfer was calculated from pre- and post-membrane blood gases as previously reported (28). Oxygenation index was defined as ([F_iO₂ x mean airway pressure] / PₐO₂). The PₐO₂ to F_iO₂ ratio (PFR) was also calculated according to the Berlin Definition (no ARDS = PFR
> 300, mild ARDS = 200 < PFR < 300; moderate ARDS = 100 < PFR < 200, severe ARDS = PFR < 100) (29). Blood urea nitrogen (BUN), postassium (K+) and creatinine were measured using an i-STAT CHEM8+ cartridge (Abott; Chicago, IL).

7.3.5 End of study procedures. End of study was initiated after 72 hrs extracorporeal circulation or upon occlusion of the ECLS circuit. Animals were irreversibly anesthetized and euthanized by exsanguination. This was done to prevent post-mortem clotting caused by routine euthanasia medications.

7.3.5.1 Fixation of explanted ECLS circuits. At the end of study, the ECLS circuit and catheter were immediately removed from the animal, rinsed with 1.5 L phosphate buffered saline (PBS) at 1 L/min flow, drained and filled with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for fixation. After 48 hours, the membrane was drained of fixative and disassembled to expose the polymethylpentene (PMP) gas exchange fibers, as we have previously described (15). A PMP fiber layer was collected from the inlet face, center and outlet face of each ML. These segments were then further dissected into 4 equal 1 cm² quadrants. Samples from the Avalon catheter were collected from the inlet and outlet connective tubing, the center of the catheter, and the catheter tip. Circuit tubing samples were collected from the pre-membrane and post-membrane lines immediately before and after the ML. All samples were dehydrated in graded ethanol (35%, 50%, 70%, 100%; 10 min each) and stored in a desiccator until imaging analysis.

7.3.5.2 Thrombus area. Digital images of the membrane lung inlet, center and outlet PMP fiber layers were captured and used to determine the percent area of thrombus deposition on each layer, which was scored by 3 blinded reviewers using ImageJ Software (NIH; Bethesda, MD, USA) as previously reported (30).
7.3.5.3 Field Emission SEM imaging. The membrane, tubing and catheter segments were sputter-coated with gold-palladium at 10 nm thickness (Leica Microsystems Inc.; Wetzlar, Germany) and imaged using field emission SEM (FEI Teneo; Hillsboro, OR, USA). An accelerating voltage of 10 kV was applied with a spot size of 9.

7.3.5.4 Protein adhesion: Protein adsorption on circuit tubing and catheters was determined as previously reported (31, 32) Briefly, following rinse with 100 mL PBS, 2 cm segments were collected in duplicated from circuit connective tubing, catheter inlet/outlet tubing connections, catheter center and catheter tip. Additional tubing samples that had not been exposed to blood were cut from the circuit before use as controls. Segments were incubated in 1 wt% aqueous solution of sodium dodecylsulfate (SDS) buffer with agitation at 37 C for 1 h. Samples were centrifuged at 200 g for 10 min to sediment red blood cells, and total protein concentration in the supernatant was determined using the Pierce™ micro bicinchoninic acid (BCA) test kit (ThermoFisher Scientific; Waltham, MA). Total protein was divided by the blood-contacting surface area of the sample.

7.3.5.5 Histology. Following euthanasia, tissue samples were taken from lungs, kidney, liver, jejunum, left ventricle and aorta for histological imaging and assessment. Samples were fixed in neutral-buffered 10% formalin for 24 hrs, trimmed, embedded in paraffin, sectioned at 4 µm, and stained with hematoxylin and eosin (H&E). Histological images were recorded using a 10x objective (Zeiss Axioskop; Oberkochen, Germany) and were evaluated by a pathologist blinded to study conditions to assess signs of toxicity, end-organ damage and diffuse alveolar damage in the lungs (33).
7.3.6 Statistics. Statistics were performed using SAS 9.4 (Cary, NC, USA). All tests were two-sided with an alpha = 0.05 for significance. The CTRL and TLP groups were tested independently using one-way mixed models with repeated measures and a Dunnett adjustment to test significant changes from baseline measurements. Between-group differences were examined using a two-way mixed model with repeated measures. A Tukey adjustment was used for multiple comparisons. Survival analysis was performed with log-rank test for significance.

7.4 Results.

We successfully completed the first multi-day evaluation of heparin-free ECLS using clinically available ECLS circuits with TLP coating. In the CTRL group, all animals survived to end of study with one circuit that occluded at the 72-hr mark. In the TLP group 4 animals died before 72 hr: two due to thrombotic circuit occlusions, one due to pneumothorax and one after rapid development of desaturation/destabilization unresponsive to vasopressors. Only one animal survived for 72-hrs circulation (for TLP at 24 hrs, n=5; at 48 hrs, n=3; at 72 hrs n=1). For TLP mean survival time was 57 ± 13 hrs versus 72 ± 0 hrs in CTRL (p = 0.026).

Complete blood count and coagulation values are shown in Table 7.1. Red cell count and hemoglobin concentration dropped significantly in both groups after start of ECLS. Platelet count significantly decreased immediately Post-ECLS in TLP and at 12 hrs in CTRL. PT was elevated in both groups during ECLS. aPTT and ACT were significantly higher in the CTRL group versus TLP after start of heparin infusion in CTRL. D-dimer concentration did not change in either group (Figure 7.2). ATIII activity was significantly reduced from 3h to end of study in CTRL and was reduced from 3-12hrs in TLP (Figure 7.2). vWF activity numerically decreased in both groups on the first day of circulation but began to increase at 24 hrs (Figure 7.2). Thromboelastography (Figure 7.3) showed that R was prolonged versus BL in CTRL and
Table 7.1 Blood Cell Count and Coagulation.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Baseline</th>
<th>Post-ECLS</th>
<th>3 hr</th>
<th>6 hr</th>
<th>12 hrs</th>
<th>24 hrs</th>
<th>48 hrs</th>
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<td>130±13†</td>
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<td>126±9</td>
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<td>3.9±0.2</td>
<td>4.1±0.2*</td>
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Mean ± standard deviation. White blood cell (WBC), red blood cell (RBC), hemoglobin (Hb), platelet (Plt), prothrombin time (PT), activated partial thromboplastin time (aPTT), activated clotting time (ACT), fibrinogen (Fib), plasma free-hemoglobin (PFHb), blood urea nitrogen (BUN), potassium (K+). *Indicates significant change from baseline. †Indicates significant difference between groups. Significance p<0.05.
Figure 7.2 Mean ± standard deviation of (A) D-dimer concentration, (B) anti-thrombin III (ATIII) activity and (C) von Willebrand factor (vWF) activity in platelet poor plasma from control animals (CTRL; n=5) receiving extracorporeal life support (ECLS) with heparin-coated circuits and continuous heparin infusion versus tethered liquid perfluorocarbon (TLP) animals (n=5) receiving ECLS using TLP-coated circuits and no systemic anticoagulation for 72 hours circulation. *Indicates significant change from baseline. †Indicates significant difference between groups. All tests were two-sided with significance p<0.05.
Figure 7.3 Mean ± standard deviation of thromboelastography measurement of (A) clot reaction time (R), (B) initial clot formation time (K) (C) clot strength (MA) and (D) fibrinolysis at 60 minutes (LY60) in whole-blood from control animals (CTRL; n=5) receiving extracorporeal life support (ECLS) with heparin-coated circuits and continuous heparin infusion versus tethered liquid perfluorocarbon (TLP) animals (n=5) receiving ECLS using TLP-coated circuits and no systemic anticoagulation for 72 hours circulation. *Indicates significant change from baseline. †Indicates significant difference between groups. All tests were two-sided with significance p<0.05.
was significantly elevated compared to the TLP group. K increased in both groups with time on ECLS. Both groups exhibited a reduction in MA after start of ECLS which returned to BL levels by 24 hours and continued to increase. LY60 was initially elevated in the TLP group Post-ECLS but was significantly reduced in both groups by 24 hrs. Normalized platelet count (Figure 7.4) was reduced at 24 hrs in TLP and 48 hrs in CTRL. Both groups exhibited an increase in platelet aggregation stimulated by collagen and ADP with time on ECLS (Figure 7.4).

All circulation-related complications and significant events are reported in Table 7.2. Both groups experienced hemorrhagic and thrombotic complications. The most common site of bleeding was peripheral cannulas; and the most common site for thrombi was the membrane. Interestingly, arterial bubbles were detected by the integrated circuit alarm in 60% of animals in the TLP group (mean onset 2 ± 3 hrs post-ECLS) which was normally resolved by 6 hrs. This did not occur in CTRL. Additionally, 80% of TLP animals required an increase in sweep gas flow rate and 60% required an increase in circuit blood flow to maintain the patient in normoxic/normocapnic state; whereas no change in sweep gas or blood flow was required in CTRL.

Post-circulation assessment of ECLS circuits revealed no difference in thrombus deposition or protein adhesion between groups. The percent area of thrombus deposition on PMP fiber sheets collected at the inlet, center and outlet of the membrane was not different between groups, but progressively decreased in both groups from the inlet/venous face to the outlet/arterial face (Figure 7.5). There was a significant decrease in thrombus area on the post-membrane face versus the pre-membrane face in the TLP group. SEM imaging indicated that thrombus deposition on TLP-coated materials with heparin-free circulation was similar to heparin-coated CTRL devices with continuous heparin infusion (Figure 7.6). Again, the largest thrombotic deposits and areas of
Figure 7.4 Mean ± standard deviation of (A) Normalized platelet count (calculated by [platelet (Plt) * (Hemoglobin concentration at specified time point / baseline hemoglobin concentration)], (B) collagen (COL)-stimulated platelet aggregation, and (C) adenosine diphosphate (ADP)-stimulated platelet aggregation in whole-blood from control animals (CTRL; n=5) receiving extracorporeal life support (ECLS) with heparin-coated circuits and continuous heparin infusion versus tethered liquid perfluorocarbon (TLP) animals (n=5) receiving ECLS using TLP-coated circuits and no systemic anticoagulation for 72 hours circulation. Platelet aggregation was measured via impedance aggregometry and reported as total area under the curve (AUC) divided by the platelet count. *Indicates significant change from baseline. †Indicates significant difference between groups. All tests were two-sided with significance p<0.05.
Table 7.2 Complications and Significant Events.

<table>
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<tr>
<th>Event</th>
<th>CTRL % (Time ± SD)</th>
<th>TLP % (Time ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemorrhage: ECLS Cannula Site</td>
<td>20% (19 h)</td>
<td>N/A</td>
</tr>
<tr>
<td>Hemorrhage: Peripheral Cannula</td>
<td>60% (23 ± 4 h)</td>
<td>20% (47 h)</td>
</tr>
<tr>
<td>Hemorrhage: Airway</td>
<td>40% (24 ± 20 h)</td>
<td>N/A</td>
</tr>
<tr>
<td>Circuit Thrombus: Pump</td>
<td>N/A</td>
<td>20% (unknown)</td>
</tr>
<tr>
<td>Circuit Thrombus: Membrane</td>
<td>40% (69 ± 1 h)</td>
<td>40% (47 ± 15 h)</td>
</tr>
<tr>
<td>Circuit Thrombus: Occlusion</td>
<td>20% (72 h)</td>
<td>40% (50 ± 18 h)</td>
</tr>
<tr>
<td>Circuit Arterial Bubble Detected</td>
<td>N/A</td>
<td>60% (2 ± 3 h)</td>
</tr>
<tr>
<td>Increase Sweep Gas</td>
<td>N/A</td>
<td>80% (39 ± 22 h)</td>
</tr>
<tr>
<td>Increase Blood Flow</td>
<td>N/A</td>
<td>60% (41 ± 21 h)</td>
</tr>
<tr>
<td>Vasopressors</td>
<td>20% (32 h)</td>
<td>80% (44 ± 18 h)</td>
</tr>
<tr>
<td>Diuretic/Furosemide</td>
<td>60% (3 ± 3 h)</td>
<td>60% (13 ± 18 h)</td>
</tr>
</tbody>
</table>

Instance of significant events during extracorporeal circulation including complications, deviations from initial circuit settings necessitated by animal condition and membrane performance, and administration of medications unrelated to anesthesia. The percent instance of each event for control (CTRL; n=5) and tethered-liquid perfluorocarbon (TLP; n=5) groups are shown. The mean time to onset of event (hours post-ECLS initiation) ± standard deviation is shown.
dense fibrin coverage were observed on the inlet/venous face and progressively decreased towards the outlet/arterial face. Thrombus deposition on the Avalon catheter was similar between groups, with greatest deposition observed at the catheter inlet and outlet connection (Figure 7.7). Of note, deposition on connective tubing was minimal or absent. There was no difference between groups in total protein adsorption on the segments collected from the Avalon catheter (Figure 7.5).

Vital signs and respiratory parameters are reported in Table 7.3. PaCO₂ was significantly reduced versus baseline in both groups at Post-ECLS, which persisted in the CTRL group until 72 hrs, but was not observed in TLP. MV was reduced in both groups following ventilator setting decrements made after the Post-ECLS time point. RR was reduced versus baseline in CTRL from 3 hrs to 72 hrs; but was only reduced in TLP from 3-12 hrs. There were no differences in VCO₂ between groups. CTRL animals were primarily supported by room air; whereas TLP animals had a significant increase in FiO₂ beginning at 12 hrs. Within hours of start of ECLS, plateau pressures were higher in the TLP group and C_{dyn} was reduced compared to CTRL. Pulmonary resistance increased in both groups versus baseline at 3 hrs to end of study. OI was significantly higher in TLP versus CTRL beginning at 12 hrs. PFR was significantly reduced in TLP beginning at 12 hrs and was significantly reduced in CTRL at 72 hrs. There were no significant differences between groups in percent reduction in pCO₂ across the membrane or oxygen transfer (Figure 7.8). There were no between group difference in ECLS circuit settings or pressures (Table 7.4).

Systemic cytokine levels (Figure 7.9) were unremarkable, except an increase in IL-6 in TLP beginning at 48 hrs. Additionally, IL-8 was significantly reduced from 12-24 hrs in both groups. There was no change in systemic syndecan-1 levels in either group (Figure 7.9). Following necropsy assessment and histological analysis, there were no obvious signs of toxic
Figure 7.5 (A) Mean ± standard deviation protein adhesion (total protein concentration / blood contacting surface area) collected from 19 Fr dual-lumen catheters for veno-venous extracorporeal life support (ECLS) follow 72 hours circulation in vivo in swine (n=5/group). Control animals (CTRL) used manufacturer’s standard catheters during ECLS with continuous heparin infusion versus tethered liquid perfluorocarbon (TLP) animals that used standard catheters with TLP-coating applied during ECLS with no systemic anticoagulation. (B) Mean ± standard deviation of total thrombus area determined from digital images of polymethyl pentene fiber layers collected from the membrane lung inlet/venous face (INLET), center layer of the membrane (MID), and outlet/arterial face of the membrane (OUTLET). The percent of the total membrane area that was covered in thrombus was scored by 3 blinded reviewers, and the average of the 3 scores was taken for each sample. *Indicates significant change versus inlet. †Indicates significant difference between groups. All tests were two-sided with significance p<0.05.
Figure 7.6 Field emission scanning electron microscopy images from membrane lungs with immobilized-heparin control coating (CTRL; panels A-F) used with continuous heparin infusion versus membranes with tethered liquid perfluorocarbon (TLP; panels G-L) coating used without systemic anticoagulation for 72 hours extracorporeal circulation in swine (n=5/group). Top row images (A-B, G-H) from the inlet/venous face of the membrane lung. Center row images (C-D, I-J) from the center of the membrane lung. Bottom row images (E-F, K-L) from the outlet/arterial face of the membrane lung. Significant thrombus deposition present on all membrane lungs, with no apparent difference between groups. Layers of fibrin mesh cover the gas exchange fibers, potentially inhibiting blood flow and gas transfer. Density and extent of thrombus formation appeared to decrease from the inlet face to the outlet face.
Figure 7.7 Field emission scanning electron microscopy images collected from segments of 19 Fr dual-lumen catheters for venovenous extracorporeal life support following 72 hours in vivo circulation in swine (n=5/group). Control catheters (CTRL; top row, panels A-E) were used with continuous heparin infusion and are compared to tethered liquid perfluorocarbon (TLP; bottom row, panels G-L) coated catheters used without systemic anticoagulation. Samples were collected from the catheter inlet connection (A, F), outlet connection (B, G), center of the catheter (C, H) and catheter distal tip (D, I). Greatest deposition in both groups was observed on the inlet and outlet connections. There was no apparent difference between groups in sample appearance.
Table 7.3 Vital Signs and Respiratory Measurements.

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<th>Variable</th>
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<th>Post-ECLS</th>
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<th>24 hrs</th>
<th>48 hrs</th>
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Mean ± sd control (CTRL) versus tethered liquid perfluorocarbon (TLP) (n=5/group. Heart rate (HR), mean arterial pressure (MAP), partial pressure oxygen and carbon dioxide in arterial blood (PₐO₂ and PₐCO₂), minute ventilation (MV), respiratory rate (RR), fraction inspired oxygen (FIO₂), endtidal carbon dioxide (Et CO₂), respiratory compliance (Cdyn), PFR (PₐO₂ to FIO₂ ratio) *Indicates significant change from baseline. †Indicates significant difference between groups. Significance accepted as p<0.05.
Figure 7.8 Figure represents gas exchange efficiency of membrane lungs with tethered liquid perfluorocarbon (TLP; n=5) coating versus immobilized-heparin control coating (CTRL; n=5). Time points are post-initiation of ECLS (PE) and at 3-72 hours after start of circulation. Panels A and B bars represent means ± standard deviation of partial pressure of carbon dioxide (pCO₂) and partial pressure of oxygen (PO₂), respectively, for blood gases drawn from the pre-membrane (PRE) venous ECLS circuit line and post-membrane (POST) circuit line located immediately before and after blood passage through the membrane lung. Panel C represents mean ± standard deviation of percent decrease in pCO₂ from pre-membrane to post-membrane blood samples. Panel D represents mean ± standard deviation oxygen transfer (ml/min) measured from blood samples directly before and after passage through the membrane lung in the extracorporeal circuit. A two-sided test was performed with p < 0.05 accepted for significance. *Indicates within group change vs. baseline; †Indicates between group difference.
Table 7.4 ECLS Circuit Parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Post-ECLS</th>
<th>3 hr</th>
<th>6 hr</th>
<th>12 hrs</th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
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<tr>
<td>Flow (L/min)</td>
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<tr>
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<td>5±0</td>
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<td>7±2</td>
<td>10±4</td>
<td>14±4†</td>
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<td>Pump RPM</td>
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<td>2630±20</td>
<td>2620±40</td>
<td>2650±40</td>
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<td>2760±40</td>
<td>2750±40</td>
<td>2730±40</td>
<td>2840±150</td>
<td>3000±170</td>
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<td>-79±9</td>
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Mean ± standard deviation in control animals (CTRL; n=5) receiving extracorporeal life support (ECLS) with heparin-coated circuits and continuous heparin infusion and tethered liquid perfluorocarbon (TLP; n=5) group receiving ECLS using TLP-coated circuits and no systemic anticoagulation for 72 hours. RPM = pump revolutions per minute, P ven = membrane inlet pressure/negative pressure, Δp = membrane pressure gradient/pressure drop. *Indicates significant change from baseline. †Indicates significant difference between groups. Significance p<0.05.
Figure 7.9 Mean ± standard deviation of systemic cytokine levels expressed as the relative quotient (RQ) from blood samples collected throughout 72 hours of extracorporeal life support (ECLS). Control animals (CTRL; n=5) received ECLS with heparin-coated circuits and continuous heparin infusion versus tethered liquid perfluorocarbon (TLP) animals (n=5) that received ECLS using TLP-coated circuits and no systemic anticoagulation. (A) Interleukin-1β (IL-1b), (B) interleukin-6 (IL-6), (C) interleukin-8 (IL-8), (D) interleukin-10, (E) tumor necrosis factor α, and (F) endothelial damage marker syndecan-1 were measured at baseline (BL), post-initiation of ECLS (PE) and at 3, 6, 12, 24, 36, 48, 60 and 72 hours post-ECLS. *Indicates significant change from baseline. †Indicates significant difference between groups. All tests were two-sided with significance p<0.05.
damage or distinctive pathologies in the lungs, kidney, liver or jejunum for either group. Findings were mostly unremarkable, with the exception that all animals had signs of lower lung consolidation likely due to positional atelectasis and mechanical ventilation (see Supp. Fig 1). Two animals in each group had signs of more severe lung damage including fibrosis, edema, accumulation of protein aggregates and type II epithelial cell damage. Additionally, one animal in each group had hepatic congestion and inflammation. Two TLP animals and one CTRL animal had signs of kidney damage (see Appendix E)

7.5 Discussion

This is the first multi-day study reporting heparin-free ELCS using TLP-coated circuits. In comparison to the standard-of-care, heparin-coated circuits and continuous heparin infusion, TLP did not offer an advantage leading to worse oxygenation and higher mortality. To our knowledge this is also the first evaluation of an investigational (not clinically available) hemocompatible coating applied to complete, standard ECLS circuits coated tip-to-tip (including membrane, blood pump, tubing and catheter) evaluated on a multi-day time frame in vivo in an ICU setting. The study closely represents the clinical care scenario which is a third unique feature of our work. We hypothesized the TLP is safe, efficacious and preserves native blood parameters during 72 hours of ECLS without supplemental anticoagulation. Our primary finding is the TLP coating alone is insufficient to prevent circuit thrombosis for 72 hours of ECLS. Additionally, we found that membrane performance was altered in the TLP group requiring higher sweep gas flow than controls (Table 7.4). Finally, we found that heparin-coated ECLS with continuous heparin infusion also does not prevent circuit thrombosis for 72 hours, and we document the impact of this therapy on healthy animals including platelet count and aggregation, thromboelastography, coagulation and hemolysis.
Blood parameters were similar in both groups with time on ECLS, and we did not notice significant preservation of native cell counts in the TLP group (Table 7.1). Red blood cell count and hemoglobin were significantly decreased in both groups by 3 hrs after start of ECLS. Shear stress from the blood pump causes hemolysis and necessitates blood transfusion in most ECLS patients (34). Interestingly, PFHb, which is used to measure hemolysis, did not increase with time on ECLS in CTRL. In TLP, PFHb was significantly higher versus CTRL beginning at 12 hours Post-ECLS; however, the average concentration was still below levels indicative of clinical hemolysis (PFHb > 50g/dL) (35), except for the one TLP animal that survived to end of study (PFHb = 68 g/dL at 72 hours). The blood pump revolutions per minute (RPMs), although not statistically significant, were consistently ~100-200 revolutions higher in the TLP group than CTRL, which could cause greater hemolysis. This seemingly minimal difference in RPMs needs to be seen as an indication of early and sustained increase in resistance within the TLP circuits, likely due to thrombus formation; and is concurrent with the trend toward elevated ∆p observed in TLP. We previously evaluated TLP-coated ECLS circuits compared to heparin-coated circuits for 6 hrs of heparin-free circulation. We noted an elevated transmembrane pressure immediately post-ECLS; however these levels declined and were the same at controls by 1-hour after start of circulation (Chapter 6). There were no differences in RPMs or other circuit parameters for the 6 hour duration of the study.

Platelet count (both concentration shown in Table 7.1 and normalized count corrected for hemodilution in Figure 7.4) was reduced with time on ECLS in both groups, and no between groups differences were observed. Platelet count is widely utilized as a measure of biocompatibility of blood contacting surfaces (36, 37) and has repeatedly been shown to decrease with time on veno-venous ECLS with heparin infusion (4). Our results show no benefit of TLP on
preservation of platelet count relative to CTRL over 72 hours. TLP has been shown to reduce plasma protein adsorption and platelet adhesion but does not address the effects of shear stress and contact pathway activation on platelet activation and aggregation, which could contribute to platelet consumption.

Fibrinogen concentration, which is normally reduced with protein adsorption and thrombus formation during ECLS, was significantly elevated in both groups between 24-72 hrs, potentially as an acute inflammatory response (38, 39). aPTT and ACT were significantly higher in the CTRL group versus TLP following start of heparin infusion; in TLP the aPTT and ACT transiently increased following the cannulation heparin bolus but returned to baseline levels shortly after and were stable throughout. Despite similar blood cell counts throughout the study, bleeding complications were more frequent in CTRL (occurred in 4 of 5 animals) and began much earlier between 19-26 hrs after start of ECLS; whereas, in TLP only 1 animal had secondary bleeding which began at 47 hrs (Table 7.2). This could suggest that the bleeding observed was primarily due to anticoagulant administration in the CTRL group. This finding is in line with reports by Andrews and the current Extra Corporeal Life Support Organization (ELSO) summary, stating that cannulation site bleeding is the most frequently reported hemorrhagic complication during adult respiratory therapy (5, 10).

D-dimer levels (Figure 7.2), which have been used as an indicator of clot formation in the membrane, were not significantly different from baseline in either group despite formation of occlusive thrombi in 3 animals; additionally, D-dimer levels in animals where circuits became occluded did not show an obvious increase prior to occlusion. Of note, all circuit occlusions were preceded by a rapid increase in in ∆p (Table 7.4). A transient decrease in ATIII levels was observed in both groups compared to baseline at 3-6 hours after start of ECLS; however, after this
point ATIII continued to decrease in CTRL animals but returned to baseline levels in TLP (Figure 7.2). Decrease in ATIII levels is often observed with time on ECLS when heparin anticoagulation is used and can require administration of supplemental ATIII (40). The anticoagulant effects of heparin occur through potentiation of ATIII activity, increasing activity nearly 1000-fold; and without adequate ATIII levels, the anticoagulant effects of heparin will decrease. The decrease in ATIII observed in CTRL was accompanied by an increased rate of heparin infusion (100,000 U / L) from Post-ECLS (7.8 ± 2.0 mL/hr) to 72 hours (33.6 ± 1.2 mL/hr). We saw a transient decrease in vWF (Figure 7.2) in both groups during the first 24 hours on ECLS, which can occur due to shear stress-induced uncoiling and cleavage of high molecular weight multimers of vWF (41) or vWF-mediated platelet adhesion in high shear environments; however baseline levels were recovered by 24 hours and there were no between-group differences.

Thromboelastography was used to evaluated coagulation in whole blood from start of clot formation to clot retraction and fibrinolysis (Figure 7.3). We observed a prolonged R time in CTRL despite the use of heparinase cups to neutralize the effects of heparin infusion. The observed increase in K may be due to hemodilution and consumption of clotting factors; and was concurrent with reduction in platelet count and increase in PT in both groups. Interestingly, clot strength (MA) which is related to platelet count and function (42), initially decreased with platelet count, but returned to baseline levels by 24 hours and continued to increase despite progressive platelet loss. This may be due to hyperactivity and aggregation of platelets, as we observed a significant increase in both ADP and collagen induced platelet aggregation at 24-48 hrs in both groups (Figure 7.4). This increase in aggregation is consistent with the findings of Prat, et al, that reported an increase in collagen and ADP stimulated platelet aggregation in healthy anesthetized sheep on veno-venous ECLS for five hours, regardless of whether the animals received standard heparin infusion during
ECLS or no continuous heparin (25). The increase in aggregation was contradictory to the findings of a recent clinical systematic review of platelet function during extracorporeal membrane oxygenation (ECMO) (43). In the reviewed studies, time on ECMO reduced or did not impact ADP-stimulated platelet aggregation. We think this discrepancy is explained by the fact that, as the authors point out, the reviewed studies do not account for the effect of platelet count on measurement of aggregation, so the reduced aggregation may be due to a significant reduction in platelet count with time on ECMO (43).

We assessed thrombus deposition on all circuit materials at the end of study to investigate the location and extent of thrombus formation. We observed substantial thrombus deposition on the membranes in both groups (Figures 7.5 and 7.6); and the membrane was the most common site of thrombus deposition, which is consistent with clinical findings (34). Additionally, we noted in both groups that thrombus deposition was most prominent on the inlet/venous face and gradually reduced toward the outlet. Dense fibrin layers with incorporated white and red blood cells and platelets were observed around the PMP fibers and likely impeded gas exchange (3). The density and area of coverage of these deposits were notably less on the outlet face of the membrane. Interestingly, in our previous study comparing TLP to heparin coating during 6 hours heparin-free ECLS, we saw notable thrombus formation and fibrin sheets on the control, heparin-coated materials that were significantly reduced or absent on TLP membranes (see Chapter Six). In this way, TLP coating may be suitable for short-term applications, but did not reduce thrombus formation over the 72 hour time-frame performed in this study.

Thrombus formation on the catheter was visibly less significant compared to the membrane. The catheter outlet and inlet were the sites where most thrombi and cellular deposition were present on the catheters (Figure 7), likely because the blood path diameter rapidly changes
at these connection points, inducing turbulent flow. This is similar to what we observed in our 6 hour evaluation of TLP-coated ECLS circuits and catheters (Chapter 6). Clot formation was minimal on the tubing samples for both groups, which is important to note as most in vitro studies only examine tubing, thereby neglecting other regions where thrombus formation can occur. We assessed protein adhesion as this method has previously been utilized to demonstrate the omniphobic properties of TLP (18). We utilized higher flow rates and a longer duration of blood exposure compared to other reports and did not observe a difference in protein deposition between TLP coated catheter and tubing segments versus CTRL (Figure 7.5). These findings, along with the instance of thrombotic circuit occlusion in both TLP and CTRL groups, not only suggest that TLP coating alone without supplemental anticoagulation is insufficient for prevention of thrombus formation for multi-day ECLS; but also suggests that the clinical standard of tethered heparin and continuous heparin infusion is inadequate.

We assessed vital signs and respiratory parameters to evaluate the safety of TLP coating and potential impact on membrane performance. While there were no differences in key vital signs between groups, specific critical differences in respiratory parameters were observed (Table 7.3). ECLS initially enabled ~50% reduction in mechanical ventilator settings in both groups, as has been reported for partial respiratory support using veno-venous extracorporeal CO₂ removal in healthy swine (20). This 50% reduction is now a standard for modern ECLS technology. In the CTRL group, ECLS enabled reduction in ventilator settings that persisted for the duration of the 72 hours, reflected in the decrease in RR and MV and sustained reduction in PₐCO₂. In TLP PₐCO₂ was only reduced immediately following start of ECLS prior to reduction in ventilator settings. Additionally, in TLP an increase in RR to baseline levels was necessary by 24 hrs; and an increase in F₁O₂ was required to maintain normoxia beginning at 12 hrs post-ECLS. TLP also had a
significantly higher oxygenation index compared to controls beginning at 6 hours. Cumulatively these findings imply that the gas exchange efficiency and respiratory support of the TLP-coated membranes was inferior to the CTRL membranes. The TLP group also had significantly higher plateau pressures and reduced compliance versus CTRL within the first 12 hours of ECLS, which could suggest pulmonary inflammation, edema and development of consolidation. In CTRL the PFR was consistent throughout the study and stayed above 300, signifying absence of ARDS. In contrast, in TLP animals PFR gradually decreased with time on ECLS and was even indicative of mild acute respiratory distress syndrome (ARDS) by the Berlin definition (mild ARDS = 200 < \text{P}_{a}O_{2} \text{ to } \text{F}_{i}O_{2} \text{ ratio} < 300) from 24-48 hrs (29). Interestingly, despite increased \text{F}_{i}O_{2} \text{ requirements in TLP, there was no difference between groups in O}_{2} \text{ transfer across the membrane lung (Figure 7.8 D)}; and the rate of O}_{2} \text{ transfer was comparable to what has been reported for membranes of similar dimensions and blood flow rates (44, 45). While there was no statistical difference in percent reduction in pCO}_{2} \text{ across the membrane, numerically the pCO}_{2} \text{ gradient (\% decrease between pre- and post-membrane blood samples) was always lower in the TLP group compared to CTRL (Figure 7.8, A and C). The sweep gas flow rate had to be increased from 5 L/min up to 10-20L/min in 80\% of the TLP animals to achieve this pCO}_{2} \text{ gradient. Taken together this suggests that gas transfer was not as efficient using the TLP membranes as it was in CTRL. Whether this decrease in performance is due to deposition of blood components and thrombi on the membrane that increase the diffusion barrier for gas exchange as others have reported (3); or due to damage to the native polymers during the coating process altering the gas permeability of the membrane itself, remains unclear – although the fact that we observed similar thrombus formation on CTRL membranes without the same alterations in membrane function may suggest the latter. This are critical findings that were not observed in our 6 hour evaluation of TLP heparin-free ECLS, due
to the short duration of the study; highlighting the importance of the multi-day testing format that we have performed here.

We evaluated systemic cytokine expression, endothelial damage marker syndecan-1 and histology to assess potential untoward systemic effects, inflammation and end-organ damage (Figure 7.9). ECMO has been shown to elicit complex inflammatory sequelae reminiscent of the cytokine storm described for systemic inflammatory response syndrome (SIRS). Foreign-surface contact and shear stress activate the coagulation and complement systems, promoting release of proinflammatory cytokines, leukocyte activation and migration, endothelial cell activation and infiltration of neutrophils causing end-organ damage (7). In CTRL, we did not see any change in cytokine expression compared to baseline, but in TLP we saw a numerical increase in interleukin-1β (IL-1b) and statistical increase in interleukin-6 (IL-6) beginning at 48 hrs. IL-1b is influential in endothelial cell activation and expression of cellular adhesion molecules that allow for neutrophil migration and infiltration, leading to end-organ damage (46). IL-6 has been previously reported to be elevated during ECLS, predictive of poor clinical outcomes (47), and associated with parenchymal damage in an animal model of veno-venous ECLS (46). While we observed a difference between groups in systemic inflammatory markers, our histological evaluation of end-organ damage did not reveal any stark differences between groups. We consistently observed lower-lung consolidation, but more severe damage such as fibrosis, edema, hemorrhage and necrosis that have been observed in clinical trials and animal studies was less frequently observed and was not specific to CTRL or TLP (46, 48). No specific evidence of toxic damage resulting from the TLP coating was observed, and we saw no change in syndecan-1 expression in either group. This important data suggests that more clinical histopathological data is needed following ECLS to elucidate the status of the inflammatory response during blood-polymer interactions.
While TLP has been previously shown to prevent thrombus formation without heparin in an extracorporeal shunt for 8 hours under low flow and in a 6 hour ECLS model, the material did not prevent occlusion when applied to complete ECLS circuits during 72 hours of testing in our study. It is possible that the shear stress induced by the blood pump can displace the lubricant layer from the surface, degrading the omniphobic properties of the surface. This is substantiated by the significant thrombotic deposition and occlusive thrombotic complications we observed. Another issue with this approach is that it only targets one component of ECLS-induced coagulopathy, which is adhesion of protein and cellular components. The coating does not address the contact pathway of coagulation activation, hemolysis, or shear-induced cellular activation that can drive systemic coagulation and inflammatory responses. The vascular endothelium incorporates numerous pro- and anti-thrombotic regulators to maintain blood fluidity and prevent inflammation. For these reasons utilization of several complementary approaches may be the best to avoid heparinization during ECLS. Along these lines, we believe a multi-factorial coating approach that incorporates not only an omniphobic surface like TLP, but also bioactive hemostatic regulators like nitric oxide (14, 15, 36, 49), which prevents platelet activation and aggregation, and direct thrombin inhibitors (16), which inhibit thrombin without a co-factor and can inhibit clot-bound thrombin, may be the ideal solution to prevent thrombotic and hemorrhagic complications during ECLS (50). Our laboratory has just begun studies involving this type of approach. Additionally, just as the endothelium is heterogenous with different regulatory mechanisms present in different locations based on tissue-specific demands, blood flow rate and lumen diameter; it is possible that the ideal coating for one component of the circuit may not be ideal for another; and a heterogenous, component-specific coating approach may be required for different ECLS applications (51, 52).
Many novel coatings are currently under development and will require a robust, pre-clinical assessment as we have performed here (53).

A limitation of this study is the relatively small sample size that was due to the number of circuits available for testing. The expense of the ECLS circuits ($12,000 per unit) makes them difficult to commit to these pre-clinical studies, making our sample size and experiment very unique for the translational research and clinical care community. The analysis we have performed here utilizing complete circuits and close replication of the clinical care scenario is extremely valuable and essential to fully evaluate the efficacy and longevity of a biomaterial for ECLS. We also did not have an adequate number of circuits to evaluate the use of immobilized-heparin coating without supplemental heparin; or to assess the performance of TLP with heparin infusion – to better decipher the effects of blood-biomaterial interactions versus systemic anticoagulation. Additionally, the circuits we evaluated were designed for low-flow (< 2 L / min) extracorporeal CO2 removal; the results we report may not be applicable for other forms of ECLS that utilize different blood flow rates. While there are subtle species differences in coagulation between pigs and humans, pigs were selected based on similar cardiopulmonary physiology to humans and are the standard for evaluation of new technology and coagulation during ECLS (54). We used healthy animals in this study to first understand the blood-biomaterial interaction without the additional variability of subject-to-subject coagulopathic response to injury; however, the results we observed may not be substantiated in injured subjects.

7.6 Conclusions.

In summary, we believe that the work we have performed here outlines a robust testing system for evaluation of hemocompatible materials for ECLS. The work we have conducted provides an evaluation of blood-biomaterials interactions utilizing all components of the ECLS
circuit, rather than isolated circuit components, at the flow rate which the device we evaluated is clinically utilized. We used an animal model and ICU setting that closely approximate clinical conditions; and we evaluated the materials on a multi-day time frame, rather than the standard 6-8 hr testing term commonly reported in the literature. We assessed not only the anti-thrombogenic potential of the coatings, but also assessed impact on device performance and systemic complications, which are vital considerations. We found that TLP currently does not enable heparin-free ECLS and may alter performance of the membrane. We also provide an informative assessment of ECLS-induced coagulopathy utilizing standard heparin-coated circuits and continuous heparin infusion in healthy subjects without underlying coagulopathy. The comprehensive platform we have developed for evaluation of biomaterials for ECLS incorporates techniques from biomaterials engineers (18, 49), prominent clinical scientists (14, 25, 26, 36, 49), and our extensive experience with round-the-clock multi-day animal ICU models of combat-relevant injury supported by ECLS (20, 55-58); putting us in a unique position to incorporate these transdisciplinary methodologies for a robust pre-clinical, translational assessment of biomaterials for ECLS. Going forward, we plan to test other novel biomaterials for ECLS utilizing this same format in order to identify a robust solution for heparin-free ECLS that is suitable for clinical application.

7.7 References.


Chapter Eight:

Conclusions and final considerations

8.1 Summary of major findings.

In this dissertation, we have outlined a systematic approach to evaluate biomaterials for extracorporeal life support (ECLS); and have demonstrated this approach through assessment of two novel hemocompatible surfaces: 1) a nitric oxide (NO)-generating metal organic framework (CuBTTri) and 2) a non-adhesive, liquid-infused surface called tethered-liquid perfluorocarbon (TLP). We evaluated these materials using three sequential testing phases. First, we evaluated the materials ex vivo in whole-blood preparations using standard hemocompatibility assays that demonstrate the efficacy of the materials according to their specific mechanisms of action. In this phase, we showed the CuBTTri reduces the time and rate of clot initiation, reduces clot strength and inhibits platelet aggregation both with and without addition of an NO-donor species ex vivo (Chapter Four). We also showed that TLP reduces rate of clot formation, clot strength and adherent clot weight ex vivo (Chapter Five).

In phase two, we evaluated TLP applied to all surfaces of a standard ECLS circuit in a pilot feasibility study and live swine model for 6 hours circulation time (Chapter Six). This phase puts our results in context with the literature where this 6 hour study duration is standard. We compared the TLP coating to standard heparin-coated circuits in healthy animals that did not receive supplemental anticoagulation. We evaluated not only thrombus formation and coagulation status of the animal, but also assessed the impact of the coating on ECLS membrane performance and systemic effects. We found that TLP reduces thrombus formation on ECLS membranes and
does not impede membrane gas exchange relative to controls; and exhibited no signs of toxic or systemic damage during the 6 hour testing timeline.

In our final stage of testing, we evaluated TLP-coated ECLS circuits on a multi-day (72 hour) time frame (Chapter Seven), as these technologies are used to provide therapy for days to weeks or even months at a time. We equated all aspects of this large animal study in an intensive care unit (ICU) setting to human standards of care, including equipment, anesthesia medications and provider interventions such as fluids, vasoactive drugs, diuretics, etc. We evaluated thrombus formation, coagulation status, membrane performance, systemic effects and histopathological evidence. We found that at the 72 hour timescale TLP does not reduce thrombus formation compared to the standard of care - use of heparin-coated circuits with continuous heparin infusion - and does not prevent circuit occlusion on a multi-day time frame. Additionally, we noted decreased gas exchange performance of the TLP-coated membranes, potentially from damage to the native polymers during the coating application process, that required higher sweep gas flow and greater mechanical ventilation support of animals in the TLP group. We also provide unprecedented data regarding the use of heparin-coated ECLS circuits with supplemental heparin administration in healthy subjects without underlying coagulopathic complications on a multi-day time-frame, reporting on frequency and time to onset of bleeding complications, alterations in native blood parameters and post-mortem histological assessment.

8.2 Contributions to the field.

The comprehensive testing approach we have developed in this study was built on the methodologies of world-class physician scientists and biomaterials engineers (1-3). We have adapted their techniques and combined them with our clinical expertise and unique round-the-
clock animal ICU care capabilities (4-7) to produce a truly translational evaluation system to rigorously assess biomaterials for ECLS prior to clinical use.

The novelty of our systematic approach is in the key principles that we emphasize throughout the testing process. We propose that the testing model 1) must incorporate all components of the device for which the coating was developed (for ECLS includes the membrane, catheter, blood pump and tubing), 2) must be functional under the flow conditions for which the device is utilized, 3) must perform for the intended duration of use of the device, 4) must not alter the performance of the device or cause untoward effects and 4) must be evaluated in vivo in an animal model that closely approximates the clinical scenario.

The first phase of evaluation, ex vivo assessment in whole-blood, is not unique to us and is the type of assessment performed by most biomaterial research laboratories. These methods do not closely replicate the clinical care scenario; however, we include this phase to emphasize that these tests are vital for understanding the basic mechanisms underlying blood-biomaterial interactions. Additionally, this phase is critical to fully characterize and optimize the coating before progression to more costly in vivo testing.

In the second phase of testing, we used techniques from established biomaterials studies such as assessment of thrombus formation, platelet count and function, and plasma coagulation times to provide a comprehensive assessment of blood-biomaterial interactions (1, 8). Where our study differed is that we utilized a large animal model with human-like cardiopulmonary physiology, a standard ECLS circuit with the coating applied to all components and a blood flow rate that would be used clinically. In standard in vivo biomaterial studies such as arterio-venous shunt models and the well-characterized rabbit thrombogenicity model, the coating is only applied to tubing segments and blood flow is driven by the animal’s circulation, at a significantly lower
flow rate than what is generated by the blood pump. Our model allowed us to account for the flow conditions and shear stress that occur in the clinical scenario, and also to assess the impact of the coating on the device performance as we incorporated the functional unit (the membrane) of the circuit.

The third phase of testing is where we truly distinguished our work from what has been reported previously. In the literature, studies that utilize ICU/human-like standards of care in large animal models of ECLS are generally focused on cardiopulmonary function, mechanical ventilation, device performance and clinical outcomes – without an intensive assessment of coagulation and biomaterials interactions (4, 9, 10). On the contrary, studies that included detailed assessment of coagulation and biomaterials performance utilize animal models that are far-removed from the clinical scenario. Here we have combined the strengths of both approaches to generate what is, to our knowledge, the most clinically-relevant model for assessment of biomaterials for ECLS, and specifically for extracorporeal partial respiratory support. Additionally, our ability to perform round-the-clock animal ICU studies allowed us to extend our assessment beyond the standard 6 hour time-frame to 72 hours. The 72 hour timeframe is a critical new paradigm in combat casualty care and mass casualty situations, and so our timeline is also particularly unique and relevant to applications in military and civilian care. We have shown through our evaluation of TLP that the study duration is crucial; as our promising 6 hour results were not substantiated at 72 hours.

8.3 Future directions.

We plan to utilize the biomaterials evaluation template we have developed in this study to assess other novel biomaterials for ECLS. Specifically, we plan to evaluate a multi-functional coating that has non-adhesive properties like TLP, but also inhibits platelet activation and
aggregation via NO-release similar to CuBTTri. This approach could prevent thrombus formation by targeting not only the protein adsorption that initiations thrombus formation on the biomaterial surface; but also combat the flow and shear-induced hyperactivation and consumption of platelets that propagates both bleeding and thrombotic complications (11). Multi-functional coatings that modulate blood interactions through multiple mechanisms such as occurs in the endothelium will likely be essential for development of a robust biomaterial solution to enable heparin-free ECLS.

8.4 References.


Appendix A:

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Appendix B:
IACUC approval for animal research
MEMORANDUM FOR Dr. Batchinsky, USAISR

SUBJECT: Institutional Animal Care and Use Committee (IACUC) Protocol Review Status

1. The revisions to protocol A-17-028 “Testing of Anti-thrombogenic Coatings for Heparin-free Extracorporeal Life Support in Swine” have been reviewed for compliance with the recommendations made at the 13 July 2017 IACUC meeting.

2. The protocol is approved for implementation. In accordance with IACUC Policy Memorandum A-07, IACUC approval will expire 3 years from the date of this memo.

3. The point of contact for this action is the undersigned at 210-539-7209.

HINOJOSA-LABORDE.CARMEN, PhD
Chair, IACUC
Bridge PTS Institutional Animal Care and Use Committee Review

Protocol Number: BRIDGEPTS-16 0001

Protocol Title: Training protocol on procedures for intensive care management

Principal Investigator: Paul Atter

The above protocol has been reviewed in accordance with the Guide for the Care and Use of Laboratory Animals. The background and procedures described in the proposal indicate that appropriate consideration has been given to the principles, standards, and guidelines for the care, handling, treatment, and use of the animals—including proper use of anesthetic, analgesic, and tranquillizing agents—as prescribed by the Guide for the Care and Use of Laboratory Animals.

REMARKS: Animals Authorized: Pts

De Novo Re-Write Date: 3 years from IACUC approval date

Chairperson/Scientist

USDA Category No:

USDA Category No:

USDA Category No:

USDA Category No:

Final Recommendation: Category: Effective Date 4/25/14

USDA Category:

1. No Pain or Distress
2. Minor or distress with analgesia
3. Pain or distress without analgesia
PROTOCOL NUMBER: BRIDGE PTS-17-08

DATE APPROVED BY IACUC: November 28th, 2017

TITLE: Testing of Anti-thrombogenic Coatings for Heparin-Free Extracorporeal Life Support in Swine

PRINCIPAL INVESTIGATOR: Andriy I. Batchinsky, MD

Andriy I. Batchinsky, MD
Research Scientist, The Geneva Foundation
210-619-7092 (o) / 210-386-1477 (c)
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ATTENDING VETERINARIAN: The Attending Veterinarian was consulted in the planning of procedures that required veterinary input, e.g., alleviated and unalleviated painful procedures, with the coordination of professional and technical veterinary support of the protocol.

Karyn Armstrong, DVM
Attending Veterinarian
361-522-6946 (o)
karyn.armstrong2@gmail.com

IACUC CHAIR: This protocol was reviewed and approved by the Institutional Animal Care and Use Committee.

Sarah Korn
Scientist, Bridge PTS
IACUC Chairperson
210-532-7344 (o) / 210-336-2792 (c)
sarah.korn@bridgepts.com
Appendix C:
Chapter 4 supplement
Supplement Figure 4.1 Powder X-ray diffraction results confirming synthesis of $\text{H}_3[(\text{Cu}_4\text{Cl})_3(\text{BTTri})_8]$ ($\text{H}_3\text{BTTri} = 1,3,5$-tris($1H$-$1,2,3$-triazol-$5$-yl)benzene) (CuBTTri). All measurements were performed at 4-350 with a step count of 1.0.
Appendix D:
Chapter 6 Supplement

Supplemental Methods

**ECLS circuit information:** The ECLS circuits utilized consisted of a ROTASSIST 2.8 centrifugal blood pump with integrated pressure gauge, a membrane lung (ML) (0.98 m² surface area) with integrated pressure sensor, and ¼” tube connections. The ML is designed for use with the CARDIOHELP System (Maquet Getinge Group; Rastatt, Germany)

**Initiation of anesthesia and instrumentation.** Following overnight fast with water available, anesthesia was induced with 6 mg/kg Telazol® (tiletamine/zolazepam) delivered intramuscularly (IM) via 21-25 gauge needle. Atropine sulphate (0.05 mg/kg) was administered IM to dry bronchiole secretions. Endotracheal intubation was performed via direct laryngoscopy using a 10 mm endotracheal tube. A 16 Fr Foley catheter was inserted trans-urethrally. Urine output was measured by the BARD® CRITICORE® Monitoring System (BARD Medical; Covington, Georgia, USA). Tidal volume and minute volume measured using a Haloscale® Standard respirometer (nSpire™ Health, Inc.; Longmont, CO, USA). The right jugular vein, and the left and right femoral arteries and veins were cannulated with 7-8.5 Fr sheath introducers (Arrow International Inc.; Reading, PA, USA) placed percutaneously with ultrasound guidance. Venous sheaths were cannulated with IV lines for administration of fluids (normal saline) using an Alaris™ MedSystem III® Multi-Channel Infusion Pump (Alaris Medical Systems, Inc.; San Diego, CA, USA). Arterial sheaths were cannulated with IV lines for monitoring arterial blood pressure and for collection of systemic blood samples.
**Initiation of ECLS.** ECLS circuits were primed according to manufacturer’s instructions using 1 L normal saline with 5000 U of unfractionated heparin. The 19 Fr dual-lumen catheter was percutaneously inserted into the right jugular vein for veno-venous (VV) ECLS. The catheter was connected to the ECLS system in an air-free, wet-to-wet fashion. Blood flow through the circuit was 1 L/min, and the sweep gas (100% O₂) was 5 L/min. Following cannulation, no heparin was administered.

**Assessment of gas exchange efficiency.** CO₂ removal by the membrane was determined by the percent decrease in pCO₂ from a pre-membrane to post-membrane blood gas at each time point. O₂ transfer was calculated from pre- and post-membrane blood gases as previously reported (S1). Blood chemistry (potassium, urea nitrogen, creatinine, hematocrit and hemoglobin) was analyzed from arterial blood samples using the i-STAT CHEM8+ cartridge (Abott; Chicago, IL, USA).

**Coagulation testing.** Prothrombin time (PT), activated partial thromboplastin time (aPTT), antithrombin III (ATIII), von Willebrand factor (vWF) and concentrations of fibrinogen and d-dimer were measured in previously frozen (-80 ºC) platelet poor plasma (PPP). PPP was prepared by dual centrifugation (3,000 g, 10 min, 4ºC) of citrated whole blood. Samples were analyzed using the Stago STA Compact Max Analyzer (Stago; Parsippany, NJ, USA). Activated Clotting Time (ACT) was measured using the Hemochron Signature Elite Whole-Blood Microcoagulation System (Accriva Diagnostics; San Diego, CA). Thromboelastography (TEG) was performed using the TEG 5000 Hemostasis Analyzer System (Haemonetics Corp., Braintree, MA) according to manufacturer’s instructions. 3.8% citrated arterial whole blood was analyzed using the citrated kaolin assay. Heparinase cups were used at all time points to neutralize heparin in the specimen following the bolus administered during cannulation. The following variables of
interest were recorded: reaction time (R), initial clot formation time (K), amplification rate ($\alpha$-angle), clot strength (MA), and lysis (Ly30). Plasma free hemoglobin was measured by direct spectrophotometry as previously described (S2).

**ICU care.** Animals were continuously monitored by technicians, study staff and a physician throughout the experiment. Isofluorane anesthesia was titrated and monitored such that the animal was anesthetized for the duration of the study while spontaneously breathing. Fluid administration was adjusted hourly to achieve a minimum 0.5 ml/kg/hr urine output. All vital signs and sensor data were recorded by manual transcription hourly. Urine output, fluid balance, anesthesia rates and rectal core temperature were also recorded.

**Application of TLP coating to ECLS circuits.** Application of TLP coating to ECLS circuits was carried out in 2 phases. In the first phase, application of the perfluorocarbon tether layer was done by Free Flow Medical Devices, LLC (Lancaster, PA, USA). Briefly, circuits were exposed to low-pressure radio frequency oxygen plasma at 100 W. A liquid silane solution was circulated by peristaltic pump through the circuit for 24h. Circuits were then rinsed and dried. The tether layer was applied to the dual-lumen ECLS cannulas in the same fashion. All circuits and cannulas were then shipped to San Antonio, TX, for testing. The second phase of coating, application of the liquid perfluorocarbon lubricant layer, was carried out on the morning of the study before cannulation. A volume of 150 µL of the liquid lubricant, FluoroLube63 (FL63), was injected directly into the pre-membrane/venous face of the membrane lung with clamps placed on the inlet and outlet tubing to ensure that the entire membrane was saturated with the liquid. Empty syringes were connected to ports on the post-membrane/arterial face to prevent pressure build-up within the oxygenator. Once the membrane was saturated with liquid, the FL63 was flushed back and forth between syringes placed on the pre- and post-membrane faces of the oxygenator to ensure
thorough coating. The clamps were then removed to allow the FL63 to drain from the oxygenator into the pump and tubing components. The tubing and pump were positioned to ensure that all surfaces were exposed to the liquid. The excess FL63 was then withdrawn from the circuit. Approximately 30 mL of lubricant remained within the circuit. The Avalon catheters with tethered perfluorocarbon layer applied were filled with FL63, then drained of lubricant immediately prior to cannulation in the TLP group.

**Histology.** Tissue samples for histology were fixed in neutral-buffered 10% formalin for 24 hrs, trimmed, embedded in paraffin, sectioned at 4 µm, and stained with hematoxylin and eosin (H&E). Histological images were recorded using a 10x objective under (Zeiss Axioskop; Oberkochen, Germany).

**Preparation of extracorporeal circuits for analysis.** Following 6 hours of extracorporeal circulation, circuit tubing was clamped adjacent to the inlet and outlet of the catheter, then cut and disconnected from the animal. Circuits were then rinsed with 1.5 L of PBS (1 L / min flow), drained of saline and filled with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for fixation. After a minimum of 48 hours of fixation, MLs were drained of fixative and disassembled from the outer plastic housing to expose the stack of polymethylpentene (PMP) gas exchange fibers as we have previously described (S3). The top PMP fiber layer from the inlet face, the layer from the center of the fiber stack, and the bottom PMP layer from the outlet face of the membrane oxygenator were collected and imaged. The inlet, center and outlet layers were then further dissected into 4 equal square quadrants. The samples were dehydrated in graded ethanol (35%, 50%, 70%, 100%; 10 min each) and stored in a desiccator until scanning electron microscopy (SEM) analysis.

The Avalon catheter was carefully removed from the subject at the end of study and flushed with 100 mL saline. Circuit tubing samples were collected from the pre-membrane and post-
membrane lines both before use (cut from the circuit after priming with saline and before connecting to the patient) and after the explanted circuit was flushed with PBS at the end of the study. Tubing and catheter samples were fixed as described for ML samples.

Circuit tubing samples were collected from the pre-membrane and post-membrane lines both before use (cut from the circuit after priming with saline and before connecting to the patient) and after the explanted circuit was flushed with PBS at the end of the study. Tubing and catheter samples were fixed as described for ML samples.

**Field emission SEM imaging and EDS mapping.** The ML, tubing and catheter segments were sputter-coated with gold-palladium (Leica Microsystems Inc.; Wetzlar, Germany) and imaged using field emission SEM (FEI Teneo; Hillsboro, OR, USA). An accelerating voltage of 5kV was applied with a spot size of 9. The weight percent fluorine on membrane segments was measured by SEM using a JEOLISM-IT100 InTouchScope (JEOL USA, Inc.; Peabody, MA, USA) equipped with energy dispersive X-ray spectrometer (EDS). Four points of interest were selected on each sample for elemental analysis, and the average of the 4 outputs was taken for each sample. The weight percent fluorine was used to assess even distribution of the TLP coating throughout the membrane and to identify potential stripping of the coating.

**Additional statistics.** For all data sets, a Shapiro-Wilk test was conducted to test the distribution of data for normality. If skewed, data was then transformed, or the nonparametric version of the test was used. For percent area thrombus deposition scores, an interclass correlation for inter-rater reliability was performed using a one-way analysis of variance. The Shrout-Fleiss reliability method was used to generate a kappa statistic the inlet, center and outlet scores to measure agreement between reviewers.
Supplemental references.


Supplemental figures.
Supp Figure 6.1 Field Emission scanning electron microscopy images collected from 19 Fr dual-lumen catheters for veno-venous extracorporeal life support following 6 hours \textit{in vivo} circulation. Control (CTRL, top row) catheters were compared to catheters with tethered-liquid perfluorocarbon (TLP) anti-thrombogenic coating (bottom row). Segments from the inlet and outlet tubing connecting the cannula to the ECLS circuit, the center of the cannula inner lumen, and the tip of the catheter were imaged. Greater deposition of cellular debris and platelets was observed on the CTRL materials versus TLP-coated materials.
Supp Fig 6.2 Scanning electron microscopy images collected from extracorporeal life support circuit connective tubing. Control (CTRL, top row) tubing was compared to tubing with tethered-liquid perfluorocarbon (TLP) anti-thrombogenic coating (bottom row). Tubing segments from the pre-membrane venous line directly before the membrane lung (Panel A) and tubing segments from the post-membrane arterial circuit line (Panel B) were collected before exposure to blood (columns A1, B1) and after 6 hours in vivo extracorporeal circulation (columns A2, B2). Minimal deposition of debris was observed on any sample in either CTRL or TLP group. Tubing samples collected after 6 hours circulation were comparable to those collected before use.
Appendix E:

Chapter 7 Supplement
**Supp Figure 7.1** Representative images from control animals (CTRL) receiving ECLS with heparin-coated circuits and continuous heparin infusion and tethered liquid perfluorocarbon (TLP) animals receiving TLP-coated ECLS without systemic anticoagulation for 72 hours circulation. CTRL images (top row, panels A-D) were comparable to TLP images (bottom row, panels F-I) in this subset of animals where tissue from the lung (A, F), kidney (B, G), liver (C, H) and jejunum (D, I) were unremarkable with no signs of toxic injury, organ damage or thrombosis/hemorrhage.
Supplemental Figure 7.2 Representative images from control animals (CTRL) receiving ECLS with heparin-coated circuits and continuous heparin infusion and tethered liquid perfluorocarbon (TLP) animals receiving TLP-coated ECLS without systemic anticoagulation for 72 hours circulation. CTRL images (top row, panels A-C) and TLP images (bottom row, panels D-F) from a subset of animals in both groups demonstrated abnormal histopathological findings. Lung (A, D) tissue from two CTRL and two TLP animals showed fibrosis, edema, accumulation of protein aggregates and cell damage. Kidney (B, E) from one CTRL and two TLP animals showed significant inflammation and cellular damage; and liver (C, F) from one CTRL and one TLP animal showed congestion and inflammation.