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Crosslinked Urethane Doped Polyester (CUPE) Biphaseic Scaffold: Potential for *In vivo* Vascular Tissue Engineering

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Abstract

In vivo tissue engineering uses the body as a bioreactor for tissue regeneration, thus placing stringent requirements on tissue scaffolds which should be mechanically robust for immediate implantation without a long *in vitro* cell culture time. In addition to mechanical strength, vascular grafts fabricated for *in vivo* tissue engineering approach must have matching mechanical properties to the target tissues to avoid compliance mismatch, which is one of the reasons for graft failure. We recently synthesized a new generation of strong and elastic biodegradable crosslinked urethane-doped polyesters (CUPE) to address the challenge of developing soft, elastic yet strong biodegradable polymers. This study evaluated the tensile strength, burst pressure and suture retention of CUPE biphaseic scaffolds to determine if the scaffolds met the requirements for immediate implantation in an *in vivo* tissue engineering approach. Additionally, we also examined the hemocompatibility and inflammatory potential of CUPE to demonstrate its potential in serving as a blood-contacting vascular graft material. Tensile strength of CUPE biphaseic scaffolds (5.02 ± 0.70 MPa) was greater than native vessels (1.43 ± 0.60 MPa). CUPE scaffolds exhibited tunable burst pressure ranging from 1500 mmHg to 2600 mmHg, and adequate suture retention values (2.45 ± 0.23 N). CUPE showed comparable leukocyte activation and whole blood clotting kinetics to poly(L-lactic acid) PLLA. However, CUPE incited a lesser release of inflammatory cytokines and was found to be non hemolytic. Combined with the mechanical properties and previously demonstrated anti-thrombogenic nature, CUPE may serve as a viable graft material for *in vivo* blood vessel tissue engineering.

Keywords

Hemocompatibility; biodegradable elastomer; scaffold; Vascular tissue engineering

1. Introduction

Native vasculature in the form of autologous arteries and veins in combination with bypass procedures remains the primary treatment modality for coronary artery and peripheral vascular disease^{1–3}. However, one third of patients requiring bypass procedures lack these conduits due to prior surgery or disease related degeneration³. Combined with the fact that cardiovascular disease still remains the primary cause of death in the United States⁴, the shortage of autologous vascular conduits highlights an urgent need for suitable substitutes.

Non-biodegradable synthetics like Dacron and ePTFE have demonstrated reasonably acceptable degrees of success as long term substitutes for larger and midsized vessels⁵.

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However, these materials produce severe thrombotic complications when used as small diameter vessel (<5mm) replacements and are rapidly occluded². Different surface modification techniques have been implemented to improve the hemocompatibility of these synthetic grafts, including denucleation⁶, albuminization⁷, radio-frequency glow discharge (RFGD) modifications⁸ and heparin immobilization⁹. In spite of these modifications, Dacron and ePTFE are unable to remain patent over long term in a <5mm configuration.

Different tissue engineering approaches have been examined for engineering a small diameter blood vessel (SDBV). Theoretically, the use of decellularized matrices of allogenic^{10,11} or xenogenic^{12,13} origin is advantageous because they preserve the micro-architecture and composition of the vascular tissue and provide site specific remodeling and regeneration⁵. Additionally, the presence of extracellular matrix would contribute to scaffold strength and make these grafts more suited to immediate implantation after cell seeding. However, studies have indicated that the ultimate tensile strength, elasticity and compliance of these scaffolds is compromised due to shrinkage caused by tissue proteoglycans loss during detergent treatment¹⁴. The resulting compliance mismatch results in intimal hyperplasia, aneurysm formation and eventually graft failure¹⁵. Naturally available biomaterials like collagen¹⁶, fibrin¹⁷, chitosan and hyaluronic acid¹⁸ have also been used with limited success because of insufficient mechanical properties¹⁹.

Synthetic polymeric matrices made of biodegradable polymers like polyglycolic acid (PGA)^{20–22}, polylactic acid (PLA),²³ co-polymers of PLA-PGA²⁴, polycaprolactone (PCL)^{25–27}, polyhydroxyalkanoates (PHA)²⁸, and biodegradable polyurethane²⁹ have been used for vascular tissue engineering. L'heureux et al also proposed and successfully demonstrated the feasibility of a novel, completely biological approach to tissue engineering vascular grafts, which comprised of wrapping sheets of cells around a cylindrical mandrel^{30,31}. Nevertheless, the success of most of these approaches has been limited by complications related to inadequate mechanical properties, thrombosis, lack of cellular remodeling and long *in vitro* cell culture times prior to implantation.

For a tissue engineered vascular graft (TEVG) to be successful, it must emulate the mechanical and biological properties of a native blood vessel³². In order to be mechanically functional, the synthetic TEVG should satisfy the following requirements (a) The TEVG should possess the strength to withstand arterial pressure and provide adequate suture retention strength^{14,33}; (b) The TEVG should be soft and elastic to be mechanically similar to native blood vessels. This is necessary to prevent compliance mismatch at the site of anastomosis, which is the one of the major reasons for graft failure³⁴. Combined with the need for reduced *in vitro* processing time, much of the research in vascular tissue engineering in recent years has concentrated on developing vascular scaffolds with the necessary strength, compliance and bioactivity for immediate implantation after cell seeding. In particular, scaffold fabrication techniques like electrospinning^{27,35–39} and phase separation⁴⁰ have been extensively used to produce compliant nano-fibrous elastic tubular scaffolds with a wide assortment of biomaterials which include polyurethane,²⁹ polydioxanone^{38,39}, blends of polycaprolactone with collagen and elastin^{27,41} and polycaprolactone-poly lactide co-polymers³⁷.

Apart from utilizing new processing techniques to match compliance between synthetic and native grafts, much effort has also been spent on the development of new synthetic, biodegradable and biocompatible elastic polymers or elastomers⁴², whose properties may be modified by varying different reaction parameters to match that of native vasculature. Biodegradable elastomers such as poly(1,8-octanediol citrate) (POC)⁴³ and poly(glycerol sebacate) (PGS)⁴⁴ exhibit excellent biocompatibility^{45,46} and hemocompatibility^{46,47}. In particular, the excellent hemocompatibility of POC was exploited to reduce thrombogenicity

and enhance endothelial cell compatibility of ePTFE grafts *in vivo*⁴⁸. In spite of excellent biological functionality, which includes reduced inflammatory cytokine expression, lower platelet activation and adhesion and reduced tendency for clot formation, POC and PGS have insufficient mechanical properties when fabricated into scaffolds.

Recently, we developed cross-linked urethane-doped polyesters (CUPEs)⁴⁹, a new generation of biodegradable elastic polymers based on POC, for vascular tissue engineering applications. The rationale behind the synthesis of CUPE was to develop a high mechanical strength elastomer, which could be translated into a mechanically robust scaffold to overcome the deficiencies of existing elastic scaffolds. In our previous work we demonstrated that under similar polymerization conditions, CUPE is almost twenty times as strong as POC. Hence, it is expected that scaffolds fabricated from CUPE will have adequate strength to withstand arterial pressures, retain sutures and be more suited to immediate implantation than POC scaffolds. Previously, we also studied the interaction of platelets with CUPE. It was found that CUPEs adhered and activated lesser platelets compared to PLLA. However, further studies aimed at exploring interaction of CUPE with whole blood and other specific blood components are necessary to demonstrate that the doping of urethane bonds in the network structure of POC does not compromise its hemocompatibility.

In the present study, we focus on characterizing the mechanical properties of CUPE vascular scaffolds and their interaction with different blood components. Specifically, the CUPE scaffolds are characterized in terms of burst pressure, tensile properties and suture retention. The influence of CUPE on leukocyte activation and subsequent cytokine expression, hemolysis and whole blood clotting has also been explored.

2. Materials and methods

2.1. Materials

LDH-Cytotoxicity Assay Kit, was purchased from Biodivision Inc. (Mountain View, CA). Reagents for flow cytometry analysis including CD42a R-phycoerythrin (R-PE)-conjugated mouse anti-human monoclonal antibodies, CD62p allophycocyanin (APC)-conjugated mouse anti-human monoclonal antibodies, CD45 APC-conjugated mouse anti-human monoclonal antibodies, CD 11b/Mac-1 PE-conjugated mouse anti-human monoclonal antibodies, Human TNF and IL-1 β Cytometric Bead Array (CBA) were purchased from Becton Dickinson Biosciences, San Jose, CA. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and used as received unless otherwise specified. Dulbecco's Phosphate-Buffered Saline (PBS, pH=7.4) was purchased from Invitrogen (Carlsbad, CA). Gluteraldehyde buffer had 2.5% (w/v) gluteraldehyde in PBS, pH=7.4. Triton-PSB buffer contained 2% (v/v) Triton X-100 in PBS, pH=7.4

2.2 Sample Preparation

CUPE was synthesized as previously described⁴⁹. Briefly, citric acid and 1, 8-octane diol (1:1.1 molar ratio) were added to a clean round bottom flask and melt polymerized to form a clear pre-polymer. The pre-polymer was purified and re-dissolved to make a 3% (w/w) solution in anhydrous 1, 4-dioxane. 1, 6-hexamethylene diisocyanate (HDI) was added to the pre-polymer solution (1:0.9, pre-polymer:HDI mol %). Stannous octoate was used as catalyst. At pre-defined time intervals, a small amount of the reaction mixture was removed for Fourier transform infrared spectroscopy (FT-IR) analysis. The reaction was allowed to progress at 55 °C, till FT-IR results indicated that all the isocyanate was used up.

2.3. Tubular Scaffold Fabrication

A biphasic CUPE scaffold was prepared as per previously described methods⁴⁵. The non-porous phase was created by dip coating a glass rod (outer diameter 3mm) in a 3% solution (wt/wt) of CUPE0.9 in 1,4-dioxane. The thickness of the coating was controlled by varying the number of coats applied. Once a desired thickness was reached, the pre-polymer coated glass rods were air dried for 12 hours and subsequently cross linked for 12 hours in an oven maintained at 80 °C. The porous phase comprised of a 3% (wt/wt) solution of CUPE0.9 in 1,4-dioxane mixed with salt (150–250 µm) in a 1:9 ratio by weight. The resulting mixture was stirred to facilitate solvent evaporation till a viscous slurry was obtained. The biphasic scaffold was prepared by casting the slurry into tubular poly(tetrafluorethylene) molds (Inner diameter = 6mm) followed by insertion of the partially polymerized non-porous phase comprising of the glass rod with the pre-polymer coats, concentrically into the mold. The entire construct was allowed to air dry and then polymerized at 80 °C for 4 days. Salt in the porous phase was leached out by incubation with DI water (Millipore Q water purification system; Millipore, Billerica, MA) for 4 days. The DI water was changed every 12 hours. The scaffold was demolded from the glass rod by swelling in an ethanol-water (50% w/w) mixture and subsequently freeze dried for 48 hours to remove all traces of water. Scaffold morphology was examined by scanning electron microscopy (SEM) (Hitachi S-3000N, Hitachi Science Systems, Ibaaki, Japan).

2.4 Mechanical Properties

Tensile testing and suture retention tests were carried out on the biphasic scaffolds. A purely porous salt leached scaffold was used as control. All mechanical testing was carried out on a MTS Insight2 mechanical tester (MTS System Corp., Minneapolis, MN) fitted with a 10N load cell (Model 569326-01, MTS System Corp., Minneapolis, MN). A sample size of n=5 was used for all the following tests.

For tensile tests, the scaffolds were cut into rectangular strips and their dimensions were recorded using a digital caliper. The samples were mounted in the testing device and elongated to failure at a deflection rate of 500 mm/min. The stress-strain curves were plotted using the measured load-displacement data. The peak stress and strain was designated as the maximum stress and corresponding strain before failure. The initial modulus was evaluated from the slope of the stress-strain curve at 10% elongation.

Suture Retention was tested as per previously described methods⁴⁰. Briefly, the scaffolds were cut into rectangular specimens with 15×6 mm (length×width) dimensions. At 2mm from the short edge of the rectangular segment, a Prolene 5-0 suture (Ethicon, Inc) was inserted and tied to form a loop. One set of clamps of the tensile tester was used to secure the sample and the second set was used to clamp and pull the looped suture at a deflection rate of 2mm/s, till suture pull out occurred. The peak load recorded was reported as the suture retention strength.

Since the non porous segment of the biphasic scaffold is responsible for the mechanical integrity of the scaffold, burst pressure testing was carried out on non porous CUPE tubes of different thickness and 3mm inner diameter, using previously described techniques⁴⁵. Briefly, one end of the CUPE tube was connected to a digital pressure gauge (VWR International) and the other end was connected to a 60ml syringe. The syringe was filled with phosphate buffered saline and mounted on a Infusion/Withdrawal pump (Harvard Apparatus Co, Inc. Millis, MA) which had been pre-programmed with an output rate of 0.67 ml/min. The burst pressure was recorded as the maximum pressure measured by the gauge before the solid CUPE tube burst.

2.5. Whole Blood Collection

All methods related to collection and handling of whole blood and blood components like were approved by the Institutional Review Board at the University of Texas at Arlington. Acid citrate dextrose (ACD) anticoagulant containing tubes were used to collect blood drawn from healthy individuals by venipuncture and used immediately.

2.6 Whole blood clotting time

Kinetic whole blood clotting time was used to evaluate the thromboresistant properties of CUPE⁵⁰. Briefly, 850 μ l CaCl_2 (0.1 M) was added to 8.5 ml ACD blood to initiate the clotting reaction. 100 μ l re-calcified blood was immediately added on top of CUPE, PLLA, TCP and glass discs and incubated at room temperature for 10, 30, and 60 minutes. At the end of incubation time, the samples were treated with 3 ml of DI water for 5 minutes to lyse the unclotted red blood cells. The hemoglobin released from lysed red blood was measured at 540 nm absorbance using a microplate reader (Infinite M200, Tecan, Switzerland). The absorbance values were plotted versus the blood contacting time.

2.7 Leukocyte activation

The activation of leukocytes was measured by flow cytometry. 100 μ l of whole blood was incubated with CUPE, PLLA, or TCP samples for 1 h at 37 °C. Following incubation, the whole blood samples were treated with 10 μ l APC-conjugated mouse anti-human CD45 monoclonal antibodies and 20 μ l PE-conjugated mouse anti-human CD11b monoclonal antibodies for 30 minutes at room temperature in darkness. CD45 is the leukocyte maker while CD11b is used as an activated leukocytes maker. The red blood cells in the whole blood were lysed using 1 $\times$ FACS lysing solution. The antibodies conjugated leukocytes were fixed with 1% paraformaldehyde and analyzed with FACSarray bioanalyzer. 5,000 leukocytes were acquired and their mean values of yellow fluorescence density (PE), corresponding to CD11b were calculated. These values were compared with that from the PLLA group, which is set to 100% as a control group.

2.8 Inflammatory cytokine release

The inflammatory cytokines IL-1 β and TNF- α were measured using Cytometric Bead Array (CBA). Briefly, CUPE, PLLA and TCP samples were treated with 100 μ l of whole blood for 1 h at 37 °C. After incubation, the blood was collected and centrifuged at 2000 g for 10 minutes. The supernatant was carefully aspirated to obtain the platelet poor plasma (PPP). Concentrations of released IL-1 β and TNF- α in the PPP were measured using Human TNF Flex Set and Human IL-1 β Flex Set (Becton Dickinson Biosciences, San Jose, CA) following the manufacturer's instructions. Briefly, two single bead populations with distinct fluorescence intensity and capture antibodies against IL-1 β or TNF- α were incubated with the PPP samples. The bead population was resolvable in the FACSarray bioanalyzer based on their position. PE-conjugated antibodies against IL-1 β or TNF- α were further incubated with the beads and analyzed with FACSarray bioanalyzer. IL-1 β and TNF- α standard of known concentration were measured for calibration. The concentration of IL-1 β and TNF- α were calculated based on the yellow fluorescence intensity (PE) and the bead numbers using the software provided with the assay.

2.9 Hemolysis

The percentage of biomaterial-mediated hemolysis is quantified using an established method⁵¹. 200 μ l of fresh ACD blood was diluted in 10 ml 0.9% saline. 200 μ l diluted blood was incubated with each polymer sample for 2 hours at 37 °C under gentle agitation. 0.9% saline diluted blood without polymer contact served as negative controls (NC), and 200 μ l whole blood diluted with DI water served as positive control (PC). After incubation,

the polymer samples (PS) were carefully removed and the blood samples were centrifuged at 1000 g for 10 min. The supernatant was transferred to a 96 well plate and the absorbance (Abs) was measured at 545 nm. Percentage hemolysis was calculated for each polymer using the following equation.

$$\% \text{ Hemolysis} = \frac{Abs_{PS} - Abs_{NC}}{Abs_{PC}} \times 100 \quad (1)$$

2.9 Statistical Analysis

All results were presented as mean $\pm$ standard deviation. Statistical significance between data sets was established with two-tail Student's t-test. A p value <0.05 was considered to be significant.

3. Results and Discussion

The need for suitable small diameter vascular replacements for cardiovascular reconstructive procedures like coronary bypass and lower limb bypass was realized as early as the 1980s^{52,53}. However, even after three decades of research, the clinical application of small diameter vascular grafts has not been achieved. One of the primary reasons for the failure of synthetic small diameter vascular grafts is their inability to mimic the complex aspects of the biomechanical nature of native blood vessels, in particular their ultimate strength or burst pressure and their viscoelasticity⁵⁴.

In addition to cellular remedies, graft maturation can also be further progressed by using biodegradable scaffolds which are inherently strong and do not rely on extracellular matrix deposition for initial strength. Such a scaffold may be seeded with autologous cells, followed by immediate implantation at the target site. In addition to providing off the shelf availability, this technique would also theoretically enable site specific remodeling of the graft *in vivo*.

Present synthetic TEVG fabrication approaches utilize the scaffold to provide initial support and a three dimensional matrix for cells to lay down ECM. At the end of graft maturation, the scaffold would have ideally degraded leaving behind a wholly biological living graft comprised of ECM proteins and cells for implantation. The cell-seeded scaffold is directly implanted at the target site, further concerns with respect to material-blood interaction need to be addressed.

From our discussion thus far, it is apparent that the success of a vascular graft is largely dependent on selecting a scaffold material which has the strength to withstand hemodynamic pressures and is also, viscoelastic in nature from a mechanical property standpoint. Additionally, the material must also be non-thrombogenic in nature to prevent graft occlusion. We were previously able to demonstrate that CUPE in its film state possessed high tensile strength and good material-platelet characteristics⁴⁹. In this study we tested and characterized CUPE scaffolds in terms of their mechanical properties and conducted further material-blood interaction studies with CUPE films to establish its eligibility as a vascular graft scaffold material.

3.1 Scaffold Morphology

A biphasic scaffold design composed of a non porous inner lumen and a concentric porous outer scaffold, as proposed by Yang et al⁴⁵ was used for evaluating CUPE scaffold properties. As previously demonstrated, this design allows the advantages of simultaneous

seeding of smooth muscle cells and endothelial cells and subsequent compartmentalization of both cell types. From Fig 1A, it can be seen that CUPE could easily be fabricated into tubular biphasic scaffolds. The inner non porous lumen had a thickness of $100.04 \pm 12.61 \mu\text{m}$, while the outer porous segment was $1362.34 \pm 30.81 \mu\text{m}$ thick. The thickness of the inner lumen could be varied by varying the number of CUPE coatings applied. The inner diameter of the scaffold was $3057.48 \pm 192.90 \mu\text{m}$ and the porous segment had pores of dimensions $221.21 \pm 26.96 \mu\text{m}$ (Fig 1B).

3.2 Mechanical Properties

As already discussed, the mechanical properties of the scaffold used for vascular reconstruction and its similarity to biomechanics of native blood vessels plays a major role in determining graft patency^{55,56}. Previous attempts at utilizing vascular substitutes has demonstrated that intimal hyperplasia at the site of the anastomosis is one of the primary causes of graft occlusion and failure^{57–59}. Although the exact causes governing hyperplasia formation are unclear, clinical evidence has suggested that improved patency rates are achievable by closely matching the mechanical properties of the bypass graft with the target vessel to be replaced⁶⁰. Hence, the vascular scaffolds should be soft and elastic to better approximate native blood vessels.

We previously found that doping urethane bonds into the polyester network of poly(diols/citrates) resulted in a material which had the biodegradable, soft and elastic nature of crosslinked polyesters and the strong mechanical strength of linear polyurethanes. The tensile strength of the resultant CUPE films varied from $14.60 \pm 1.00 \text{ MPa}$ to $41.07 \pm 6.85 \text{ MPa}$ and corresponding break elongations of up to $337.00 \pm 6.00\%$ were obtained under the synthesis conditions investigated⁴⁹.

Tensile tests were conducted on salt leached CUPE scaffolds and biphasic scaffolds to determine if they were strong enough to function as vascular grafts. As seen from Fig 2A, the presence of a non porous thin film made the biphasic CUPE scaffold significantly stronger than the CUPE salt leached scaffold. Peak tensile stress values of the biphasic scaffold and the salt leached scaffold were $5.02 \pm 0.70 \text{ MPa}$ and $0.57 \pm 0.18 \text{ MPa}$ respectively. Similar tensile tests previously conducted on the different layers of coronary arteries revealed that the adventitial layer of these vessels had ultimate tensile stress values of $1.43 \pm 0.60 \text{ MPa}$ ⁶¹. In addition to higher tensile properties, the non porous phase of the biphasic scaffold was also responsible for a higher elastic modulus (Fig 2B) and decreased elasticity (Fig 2C), as compared to the CUPE salt leached scaffold. Moreover, the elastic modulus of these biphasic scaffolds ($3.45 \pm 0.90 \text{ MPa}$) was similar to that of radial arteries ($2.68 \pm 1.81 \text{ MPa}$)⁶². The stress-strain curves of scaffolds are characteristic of elastomer (Fig 2D). Since, the elastic modulus of elasticity provides information directly related to properties of the vessel wall, the close matching of this parameter between the scaffold and target site is essential for compliance.

The burst pressure of a TEVG is a measure of vessel strength and is therefore a key parameter which determines the suitability of a vascular graft for implantation⁶³. Since all resistance to arterial blood pressure in the biphasic scaffold is mainly provided by the non porous segment, burst pressure tests were conducted with solid non porous CUPE tubes. Tubular burst pressure was found to increase with increasing thickness of the non porous tubes (Fig 3). For similar polymerization conditions and tube dimensions, non porous CUPE tubes had higher burst pressure values than non porous POC tubes⁴⁵. CUPE tubes with wall thickness $160.58 \mu\text{m}$ had burst pressures in excess of 1500 mmHg , whereas POC tubes with thickness in excess of $400 \mu\text{m}$ had burst pressure values below 1000 mmHg . CUPE tubes with $383.96 \mu\text{m}$ wall thickness exhibited a burst pressure value of $2602.50 \pm 157.68 \text{ mmHg}$. It has been reported that saphenous veins and mammary arteries, which are the current “gold

standard” vascular prostheses have burst pressure values of 1599 ± 877 mmHg⁶³ and 4225 ± 1368 mmHg⁶⁴ respectively. Thus, by simply varying the thickness of the internal non porous segment, we could fabricate vascular grafts which accurately match the burst pressures of the vessels being replaced.

For implantation, in addition to demonstrating sufficient burst pressure, it is critical that the conduit can be sutured¹³. Both CUPE scaffolds and biphasic scaffolds had adequate suture retention strength values of 1.96 ± 0.31 N and 2.45 ± 0.23 N respectively (Fig 4). These values are significantly higher than the reported values of 1.20 ± 0.23 N required for suturing arterial vascular grafts⁶⁴ and provide further evidence that CUPE biphasic scaffolds possess sufficient mechanical properties to be implanted directly without any long term cellular re-modeling *in vitro*.

3.3 Whole Blood Clotting Kinetics

Formation of a blood clot or coagulation is a complicated process which involves the activation of a cascade of coagulation factors. The intrinsic pathway of coagulation is initiated by surface mediated reactions and hence, determining the rate of clot formation after material contact enables us to evaluate the tendency of the material to promote thrombus formation. Evaluation of whole blood clotting kinetics also assumes significance when it is taken into account that, coagulation, platelet activation and subsequent inflammation are interdependent processes. Higher absorbance values in this assay indicated that lesser number of red blood cells were involved in clot formation and therefore, corresponded to reduced coagulation. As shown in Figure 5, the absorbance measured from whole blood treated with PLLA, CUPE, glass and TCP, kept decreasing till 60 minutes after material contact, indicating the formation of an incomplete thrombus. On the other hand, complete thrombus formation took place within 30 minutes on glass. Compared to PLLA, no significant difference was found, in the clotting rate with respect to CUPE at each time point ($p > 0.05$, $n=8$). This indicated that CUPE has similar to or a little better thromboresistant properties than PLLA, which is a FDA approved material. The thromboresistant properties of CUPE may be attributed to uses of citric acid, which is used as a commercial anticoagulant, as one of the monomers in the polymer network.

3.4 Leukocyte Activation

Implanted biomaterials elicit an acute inflammatory response, due to the irreversible adsorption of fibrinogen on the material surface; this leads to the attraction of and interaction with inflammatory cells on the implant⁶⁵. Activated leukocytes show the up regulation of several distinct membrane protein receptors which allow them to bind to platelets and endothelium during the inflammatory process⁶⁶. CD11b macrophage-1 antigen (Mac-1) is a membrane glycoprotein expressed on activated leukocytes, including lymphocytes, monocytes, granulocytes, and a subset of natural killer (NK) cells. CD11b functions in cell-cell and cell-substrate interactions, mediates inflammation by regulating leukocyte adhesion and migration, and has also been implicated in immune responses such as phagocytosis, cell-mediated cytotoxicity, chemotaxis and cellular activation⁶⁷. The expression of CD11b/Mac-1 was used to determine the degree of leukocyte activation⁶⁸. In our study, no significant difference in CD11b expression on leukocytes was found between CUPE and PLLA, suggesting that CUPE could have similar inflammation as PLLA when implanted (Fig 6). The inflammation responses were also assessed by measuring the concentration of inflammatory cytokines.

3.5 Cytokine (IL-1 β and TNF- α) release

The inflammatory response generated by a biomaterial is also dependent on cytokines, which serve as signaling proteins. Cytokines are produced by a wide variety of

hematopoietic and non-hematopoietic cell types. Tumor necrosis factor alpha (TNF- α) and interleukin 1 beta (IL-1 β) are two of the best known pro-inflammatory cytokines. In the absence of exogenous stimuli, they are primarily released by activated monocytes and macrophages, at low concentrations in the blood. However, upon activation, there is an increase in the synthesis and release of these cytokines from the leukocytes. The higher cytokine concentration goes on to further influence the inflammatory process⁶⁹. Both, TNF- α and IL-1 β are associated with the up regulation of complimentary adhesion molecules on the vascular endothelium and leukocytes in the vicinity of vascular damage. Measured concentration of these cytokines has been extensively used previously as hemocompatibility markers^{47,51,70}. In this study, CUPE exposed blood samples had significantly lower concentration of TNF- α (4.89 ± 1.52 pg/ml, $p < 0.05$, $n = 7$) compared to PLLA treated blood samples (10.15 ± 3.03 pg/ml) (Fig 7a). PLLA samples also released a higher concentration of IL-1 β (11.58 ± 1.86 pg/ml) compared to CUPE samples (5.48 ± 2.16 pg/ml, $p < 0.05$, $n = 7$) (Fig 7b).

The degree of leukocyte activation combined with the release of cytokines provides an insight into the ability of the polymer surfaces to initiate an inflammatory response. Although flow cytometry analysis revealed similar degrees of leukocyte activation, the lower cytokine concentrations indicate that a less intense inflammatory response is elicited by CUPE compared to PLLA.

3.6 Hemolysis

Hemolysis analysis was conducted to quantify the potential of CUPE to damage red blood cells. The concentration of which was released by damaged red blood cells was measured photospectrometrically and was correlated to material toxicity on the red blood cells⁷¹. As shown in Figure 8, biomaterial-mediated hemolysis was found to be $< 0.5\%$ for both CUPE and PLLA, with no significant difference between them. Neither CUPE nor PLLA were observed to promote hemolysis. The results were similar to the previously reported POC that POC also does not promote hemolysis⁵¹. Taken the above hemocompatibility evaluation of CUPE together, it could be concluded that doping urethane into polyester network did not compromise the hemocompatibility of polymers.

4. Conclusion

In this focused study, we assessed the mechanical properties and hemocompatibility of CUPE to evaluate effectiveness as a prospective vascular graft material. Specifically, the mechanical properties of CUPE scaffolds in terms of their tensile strength, burst pressure and suture retention were studied. CUPE scaffolds were found to exhibited similar tensile strength, tunable burst pressure and suture retention properties as native veins and arteries. We further evaluated the hemocompatibility of CUPE *in vitro*, by assessing blood clotting characteristics, leukocyte activation, inflammatory cytokine release and red blood cell hemolysis. The data collected showed that CUPE is less prone to thrombosis and inflammation, compared to PLLA. CUPE also does not trigger severe hemolysis. The vessel like mechanical properties combined with the reduced tendency to cause thrombosis makes CUPE an excellent candidate for immediate implantation in *in vivo* vascular tissue engineering. Future studies include assessing the effect of CUPE on blood in a dynamic flow setting and evaluating the behavior of CUPE tubular scaffolds *in vivo*.

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Fig 1. Cross-section of CUPE biphasic scaffold with non porous inner phase and porous outer phase (A); High magnification image showing pore structure of porous phase and seamless integration of the two phases (B). Magnification: (A) 20x; (B) 80x.

Fig 2.

Comparison of mechanical properties of salt leached CUPE scaffold with CUPE biphasic scaffold under similar polymerization conditions (80° C, 4days). Biphasic scaffold demonstrated higher tensile stress (A), modulus (B) and lower elongation (C). The stress-strain curves of CUPE biphasic scaffold and salt-leached scaffold are characteristic of elastomers (D). N=6 for both biphasic scaffold and control. ** corresponds to $p < 0.01$.

Fig 3. Effect of non porous tubular wall thickness on measured burst pressure of CUPE tubes (80° C, 4days) (N=4–8). Burst pressure of tubes increased with increasing wall thickness.

Fig 4. Suture retention strength of biphasic CUPE scaffolds. Salt leached CUPE scaffold served as control. ** corresponds to $p < 0.01$. $N=5$ for both biphasic scaffold and control.

Fig 5.

The effect of CUPE, PLLA, TCP and glass on initiating clotting in whole blood at different time points. Higher absorbance corresponds to greater number of free blood cells and hence lesser tendency of the material to initiate thrombus formation. * and ** correspond to $p < 0.05$ and $p < 0.01$ respectively, compared to PLLA.

Fig 6. Assessment of leukocyte activation in whole blood by measuring MAC-1 antigen expression using flow cytometry. $p < 0.05$ relative to PLLA is represented by '*'. # corresponds to a p value > 0.05 . $N = 8$ for all groups.

Fig 7.

The amount of TNF- α (A) and IL-1 β (B) released by leukocytes upon incubation of whole blood with PLLA, CUPE and TCP samples, as determined by flow cytometry. * corresponds to $p < 0.05$ compared to PLLA and ** indicates a $p < 0.01$ compared to PLLA.

Fig 8.

Percentage hemolysis of red blood cells after incubation of whole blood with PLLA and CUPE discs. Blood samples which did not contact any polymer served as negative control (NC) and completely hemolysed blood in DI water was the positive control (PC). ** corresponds to a $p < 0.01$ compared to positive control. $N = 8$ for all groups.