Hemocompatibility evaluation of poly(diol citrate) *in vitro* for vascular tissue engineering

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Abstract: One of the ongoing challenges in tissue engineering is the synthesis of a hemocompatible vascular graft. Specifically, the material used in the construct should have antithrombogenic properties and support the growth of vascular cells. Our laboratory has designed a novel biodegradable, elastomeric copolymer, poly(1,8-octanediol citrate) (POC), with mechanical and degradation properties suitable for vascular tissue engineering. The hemocompatibility of POC *in vitro* and its ability to support the attachment and differentiation of human aortic endothelial cell (HAEC) was assessed. The thrombogenicity and inflammatory potential of POC were assessed relative to poly(l-lactide-*co*-glycolide) and expanded poly(tetrafluoroethylene), as they have been used in FDA-approved devices for blood contact. Specifically, platelet aggregation and activation, protein adsorption, plasma clotting, and hemolysis were investigated. To assess the inflammatory potential of POC, the release of IL-1β and TNF-α from THP-1 cells was measured. The cell compatibility of POC was assessed by confirming HAEC differentiation and attachment under flow conditions. POC exhibited decreased platelet adhesion and clotting relative to control materials. Hemolysis was negligible and protein adsorption was comparable to reference materials. IL-1β and TNF-α release from THP-1 cells was comparable among all materials tested, suggesting minimal inflammatory potential. POC supported HAEC differentiation and attachment without any premodification of the surface. The results described herein are encouraging and suggest that POC is hemocompatible and an adequate candidate biomaterial for *in vivo* vascular tissue engineering. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 82A: 907–916, 2007

Key words: hemocompatibility; biomaterials; tissue engineering; citric acid; elastomer

INTRODUCTION

Cardiovascular disease is the leading cause of mortality in the western world, with 1 million deaths annually in the United States.[1] Approximately 500,000 coronary artery and 100,000 upper and lower limb bypass procedures are performed yearly to address heart attacks and side effects due to peripheral artery disease.[1] These procedures typically involve the use of an autogenous small-diameter blood vessel (<6 mm) such as the saphenous vein. However, in 30% of patients, autogenous blood vessels are either unavailable due to prior removal or cannot be used because of preexisting vascular disease.[2] To address this problem, surgeons use synthetic vascular grafts made from expanded poly(tetrafluoroethylene) (ePTFE) or polyester (Dacron™) and researchers are investigating tissue engineered vascular grafts. The use of synthetic grafts for the replacement of small diameter vessels (<6 mm) remains a challenge as the patency rates of synthetic grafts are at most half of those obtained with vein grafts.[3] Tissue engineering, although promising, requires further research into the cell source, biomaterials, and scaffold design to successfully engineer small diameter blood vessels.

Poly(diol citrates) are a novel class of polyester elastomers that may be suitable for vascular engineering owing to their reported biocompatibility and wide range of mechanical and degradation properties.[4] Specifically, poly(1,8-octanediol citrate) (POC), has recently been proposed for use as a biphasic

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scaffold for tissue engineering blood vessels and to improve the hemocompatibility of ePTFE vascular grafts.[5,6] However, the hemocompatibility of POC has not been fully investigated. Hemocompatibility characteristics will be especially important if an *in vivo* tissue engineering approach is pursued since the remodeling graft may have direct contact with flowing blood. Therefore, the present study is a preliminary report on the hemocompatibility of POC *in vitro*. This work includes the interaction of POC with human whole blood, plasma, and monocytic cells in an attempt to capture clotting and inflammatory processes. Endothelial cell attachment and differentiation will be critical to the development of a functional endothelium, which could aid in the maintenance of graft patency. Therefore, endothelial cell adhesion and retention under physiological fluid flow conditions are also assessed.

EXPERIMENTAL

Buffers and cells

Phosphate-buffered saline (PBS) contained 137 m*M* NaCl, 2.7 m*M* KCl, 10.1 m*M* sodium phosphate dibasic, and 1.8 m*M* potassium phosphate monobasic, pH = 7.4. The platelet-suspending buffer (PSB) contained 137 m*M* NaCl, 2.7 m*M* KCl, 0.4 m*M* sodium phosphate monobasic, 5.5 m*M* dextrose, 10 m*M* HEPES, 2.5 m*M* $CaCl_2$, 0.1U/mL apyrase, 1.0 m*M* $MgCl_2$, and 4 mg/mL BSA, pH = 7.4. The Triton-PSB buffer contained 2% (v/v) Triton X-100 (Sigma–Aldrich, St. Louis, MO) in PSB, pH = 7.4. The glutaraldehyde buffer contained 2.5% (v/v) glutaraldehyde in PBS, pH = 7.4. Staining solution contained 45 mL each of methanol and water, 10 mL glacial acetic acid, and 100 mg of naphthol blue black; wash solution contained 90% methanol, 8% water, and 2% acetic acid; eluent solution contained 50% ethanol and 50% 50 m*M* NaOH with 0.1 m*M* ethylenediaminetetraacetic acid (EDTA). Human aortic endothelial cells (HAEC) were purchased from Cambrex (Rockland, ME). HAECs were grown in complete endothelial cell growth media (EGM-2, Cambrex). Undifferentiated THP-1 cells were purchased from American type culture collection (ATCC, Rockland, MD) and grown in suspension with complete RPMI-1640 media (containing 10 m*M* HEPES, 1 m*M* sodium pyruvate, 4.5 g glucose/L, 1.5 g sodium bicarbonate/L, 10% newborn calf serum, and 0.05 m*M* 2-mercaptoethanol). All chemicals were purchased from Sigma–Aldrich, unless otherwise specified.

Preparation of samples

The synthesis of poly(1,8-octanediol citrate) (POC) has been previously described.[5,7] Briefly, equimolar amounts of citric acid and 1,8-octanediol were melted together at 160°C while stirring for 15 min. The temperature was subsequently decreased to 140°C and the mixture was stirred for 1 h to obtain a POC prepolymer (pre-POC). The pre-POC was purified by precipitation in water and freeze-dried. The POC film was prepared by casting 30 wt % of pre-POC in 1,4-dioxane into a glass dish. After solvent evaporation in air at room temperature, the dish containing the pre-POC film was put into an oven at 80°C for 4 days. The resulting film was peeled off carefully and stored in a desiccator until further use. The ePTFE samples were donated by Bard Peripheral Vascular OEM Products (Temple, AZ). A transparent poly(lactide-*co*-glycolide) (PLGA 85/15) (Sigma–Aldrich) film was prepared by dissolving PLGA 85/15 in dichloromethane, which was cast into a poly(tetrafluoroethylene) (PTFE) mold. The solvent was evaporated at room temperature, producing a PLGA film. The film was removed from the mold and dried under vacuum at room temperature for 48 h. The thickness of all polymer films was 0.8 mm. Samples were cut into 10- and 5.5-mm-diameter disks using a cork borer with a surface area of 78.5 and 23.8 mm^2, respectively. The total surface area of ePTFE is greater than that of the other polymers considered here because of its surface topography; however, it was used in this study as a reference because it is the current standard-of-care regarding the use of synthetic grafts.

Preparation of platelet-rich plasma

Blood was drawn from healthy adult volunteers by venipuncture into acid citrate dextrose anticoagulant (ACD, Solution A; BD Franklin Lakes, NJ). The methods used to collect and prepare the platelets used in this study were approved by the institutional review board and the Office for the Protection of Research Subjects of Northwestern University. Platelet-rich plasma (PRP) was prepared as previously described.[8] Briefly, whole blood was centrifuged at 250 g for 15 min and the platelet-rich supernatant was removed. Plasma proteins were separated from the platelet fraction utilizing size exclusion chromatography. The columns (Bio-Rad, Hercules, CA) were packed with sepharose 2B (Sigma–Aldrich) and equilibrated with PSB. The PRP was run through the column and the elution volumes were collected and the platelet concentration was determined prior to incubation with samples.

Quantification of platelet adhesion

Glass, POC, PLGA, and ePTFE samples were incubated with the PRP for 1 h at 37°C under static conditions. The suspension was aspirated and each well was rinsed carefully three times with PBS. The number of adherent platelets was determined by detecting the amount of lactate dehydrogenase (LDH) present after cell lysis as previously described.[8,9] Briefly, adherent platelets were lysed by incubation with 2% Triton-PSB buffer for 30 min at 37°C. A colorimetric substrate for LDH (Roche Diagnostics Corporation, Indianapolis, IN) was added and incubated for 20 min at 37°C. The reaction was stopped with the addition of 1*N* hydrochloric acid. The optical density was measured at 490 nm with a reference wavelength of 650 nm. A calibration curve was generated from a series of serial dilutions of a known platelet concentration and used to

determine the number of adhered platelets. The morphology of adhered platelets was assessed via scanning electron microscopy (SEM). Briefly, adherent platelets were fixed using 2.5% glutaraldehyde in PBS for at least 2 h, dehydrated in a graded series of ethanol, and freeze-dried. The samples were then sputter-coated with a 7-nm layer of gold and observed using scanning electron microscopy (SEM 3500N, Electron Probe Instrumentation Center, Northwestern University).

Quantification of platelet activation via detection of soluble P-selectin

Glass cover slips, PLGA, POC, and ePTFE samples were incubated with 200 μL of whole blood for 1 h at 37°C under static conditions. The blood was transferred to a 1.5-mL tube and EDTA was added to a final concentration of 10 m*M*. The sample was subsequently centrifuged at 2000*g* for 10 min to obtain the platelet-poor plasma (PPP).[10] The concentration of sP-Selectin levels in the plasma was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Human soluble p-selectin Immunoassay, R&D Systems, Minneapolis, MN).

Measurement of plasma recalcification profiles

Blood was drawn from healthy adult volunteers into ACD tubes (as described earlier) and spun at 2000*g* in order to obtain the PPP. PLGA and POC samples were placed in a 96-well plate, covering the entire bottom surface of the dish, and 100 μL of citrated PPP was added to each well. Controls consisted of tissue culture-treated plastic (TCP) exposed to PPP with and without $CaCl_2$. Following the addition of PPP, a 100-μL volume of 0.025*M* $CaCl_2$ was added to each well (except the no Ca^{+2}, negative control). The plate was then immediately placed in a 96-welled plate reader, where the kinetics of the clotting process due to recalicification were monitored by measuring the absorbance at 405 nm (every 30 s for 45 min) at 37°C. In calculating the mean absorbance at each time point, 6 wells were averaged per sample. The slope of the linear portion of each profile and the clotting time to reach half maximal absorbance were calculated and analyzed. Because of the opaque nature of ePTFE, it was not tested with this method.

Quantitation of inflammatory potential via release of tumor necrosis factor α and interleukin-1β

THP-1 cells (human acute monocytic leukemia cell line) were thawed and grown in suspension in 5mL of complete RPMI-1640 media for several days at 37°C, 5% CO_2.The cells were collected and concentrated (10^6 cells/mL). A 1-mL cell suspension was placed in each well of a 48-well tissue culture plate containing PLGA, POC, ePTFE, TCP, and lipopolysaccharide (LPS, 10 ng/mL). THP-1 cells were incubated with the samples at 37°C for 18 h under static conditions. Cells were pelleted and the supernatant was removed. The concentrations of TNF-α and IL-1β in the supernatants were determined by sandwich ELISA according to the manufacturer's instructions (R&D Systems).

Hemolysis

Samples of PLGA, POC, and ePTFE were cut into 10-mm-diameter pieces and placed into clean 1.5-mL conical tubes. ACD anticoagulated blood from human volunteers was diluted (200 μL ACD blood in 10 mL 0.9% saline) and added to the tube (200 μL per tube). As a positive control for hemolysis, 200 μL of blood was diluted in sterile distilled water; whereas, saline diluted blood added to an empty tube served as a negative control. Diluted blood was incubated with samples under gentle agitation for 2 h at 37°C. Samples were then removed and each tube was centrifuged at 1000*g* for 10 min. The supernatant from each tube was transferred to a well in a 96-well plate where the absorbance was measured at 545 nm. The percent hemolysis was calculated as follows[11]:

$$\%\,\text{Hemolysis} = \frac{(\text{Absorbance of test polymer}) - (\text{Absorbance of negative control})}{\text{Absorbance of positive control}} \times 100 \quad (1)$$

Protein adsorption

The amido black assay[12,13] was used to assess protein adsorption, using fibrinogen as a model protein. Briefly, a 3-mg/mL fibrinogen (Sigma) solution was prepared in PBS. Samples of PLGA, POC, ePTFE, and nitrocellulose (Amersham HyBond C, GE Healthcare, Piscataway, NJ) were cut into 5.5-mm-diameter pieces and placed in a 96-well plate, with low protein binding plastic (Becton Dickinson, Franklin Lakes, NJ) serving as an additional negative control. The test and control samples were incubated with 200 μL of the fibrinogen solution for 1 h at 37°C. The samples were then rinsed with sterile water and incubated with the staining solution for 3 min. Following incubation, the samples were rinsed three times with wash solution and then rinsed with sterile water. The dye bound to the surface was then removed in the eluent solution under agitation for 30 min at 300 rpm. A 100-μL sample from each well was transferred to a new 96-well plate where the absorbance was measured at a wavelength of 620 nm using a 450 nm as the reference wavelength. A standard curve of known concentrations of fibrinogen was used to calculate the amount of protein adsorbed to each surface.

Cell differentiation on POC

Wells of a 12-well tissue culture plastic plate were coated with a thin film of purified pre-POC. The POC-coated plates were postpolymerized at 60°C for 7 days and extensively washed prior to cell seeding. Human aortic endothelial cells were cultured in the POC-coated wells in endothelial cell growth media-2 (EGM-2), supplemented

with 5% FBS, and human fibroblast growth factor, vascular epidermal growth factor, human recombinant insulin-like growth factor (R3-IGF-1), ascorbic acid, gentamicin, amphotericin, hydrocortisone, and heparin. To confirm the endothelial cell phenotype when cultured on POC, HAECs were probed with antibodies to endothelial cell specific markers von Willebrand Factor (vWF) (DakoCytomation, Carpenteria, CA), and vascular endothelial-Cadherin (VE-Cadherin) (Sigma–Aldrich, Milwaukee, WI). Detection of positive staining was done using the Vector lab-ABC-AP kit (Vector labs, Burlingame, CA), and the vector lab alkaline phosphatase substrate blue kit. Nuclei were counterstained with nuclear Red (Vector labs). Light microscopy was used to document the staining process.

Effect of fluid shear stress on endothelial cell adhesion

Thin films of POC were coated onto glass slides (75 mm by 38 mm, Fisher Scientific, Pittsburgh, PA) that were activated according to methods described previously.[14–17] Activated glass slides were prepared by briefly exposing the slide to a Bunsen burner flame followed by a series of incubations in 0.1*N* NaOH for 10 min, 3-aminopropyltrimethoxy silane (Sigma–Aldrich) for 5 min, and 0.8% glutaraldehyde solution (Sigma–Aldrich) for 30 min. The thin film of 30% prepolymer solution in dioxane was postpolymerized at 120°C under vacuum (2 Pa) for 3 days. Glass slides without polymer films were used as controls for the fluid shear stress experiments. HAECs between passages 2 and 5 were seeded onto POC-coated or uncoated glass slides at a density of $\sim 10.5 \times 10^3$ cell/cm^2 (30×10^4 cells in total). Seeded HAECs were cultured under static conditions for $\sim$48 h in EGM-2 and maintained in a water-jacketed incubator equilibrated with 5% CO_2 at 37°C to create confluent cell monolayers on either POC-coated or uncoated glass slides.

The CytoShear Flow Chamber System (Cytodyne, La Jolla, CA) was used to expose HAECs cultured on POC and glass slides to fluid shear stress, as previously described.[18] The entire flow system setup was kept in the water-jacketed incubator equilibrated with 5% CO_2 at 37°C. The flow rate needed for the laminar physiologic shear stress of 20 dyn/cm^2(in Ref. [18]) was calculated using the equation $Q = \tau W h^2/6\mu$. The variable Q is the media flow rate (mL/min), τ is the shear stress value of interest (i.e. 20 dyn/cm^2), W is the channel width of the chamber (2.3 cm), h is the channel height (254 μm), and μ is the fluid viscosity (assuming media has the viscosity of water: 0.01 dyn s/cm^2). All flow system components were sterilized with ethylene oxide gas. HAECs were exposed to fluid shear stress for $\sim$1 h.

The number of cells attached to the surfaces was determined with the pico green DNA assay (Molecular Probes/Invitrogen, Carlsbad, CA). Percent adhesion was calculated as the quantity of DNA from cells remaining attached on the surface after fluid shear stress divided by the quantity of DNA from attached cells under static conditions. An average of at least three slide preparations for each substrate cultured under static conditions was used for comparison.

Statistical analysis

The results were analyzed using one-way ANOVA followed by a Newman-Keuls multiple comparison test, in which a $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Platelet adhesion and activation

Platelet spreading and aggregation are markers of platelet activation[19] and have been linked to thrombotic events.[20] Therefore, in assessing the hemocompatibility of POC, we examined the morphology of platelets when in contact with POC relative to PLGA, ePTFE, and glass. Representative SEM images show platelets adhered and spread on glass, ePTFE, and PLGA [Fig. 1(a–c)]. In contrast to these reference materials, platelets incubated on POC had very low attachment and displayed a rounded morphology [Fig. 1(d)]. This observable rounded morphology upon exposure to POC suggests that the platelets are not activated. The number of platelets that adhered to each test surface was quantified by measuring LDH activity. A significantly lower number of platelets adhered to POC and ePTFE than to either glass or PLGA (Fig. 2, $p < 0.01$). There is no significant difference in the number of platelets attached to PLGA (224,481 ± 40,103 mm^{-2}), when compared with glass (259,468 ± 40,442 mm^{-2}). The number of platelets on POC (40,045 ± 8,210 mm^{-2}) was approximately a third the number of platelets that attached to ePTFE (126,454 ± 18,213 mm^{-2}). When assessing the antithrombogenic potential of a surface, in addition to platelet attachment, platelet activation should also be considered. It has been shown that although the surface of a material may not support significant platelet adhesion, platelet activation may still occur.[20,21]

Once activated, platelets undergo biochemical and morphological changes, such as exocytosis of granule contents, which includes the release of soluble P-selectin.[22] The measure of soluble P-selectin is commonly used as an indicator of platelet activation.[23–26] Exposure of platelets to glass resulted in the release of significantly more sP-selectin relative to POC and ePTFE ($p < 0.01$) (Fig. 3). Significantly more sP-selectin was detected from samples incubated with PLGA relative to POC ($p < 0.001$), suggesting that the platelets exposed to PLGA and glass were more activated (Fig. 3). These data show that POC does not promote significant platelet adhesion or activation, which are the first steps in thrombus formation.

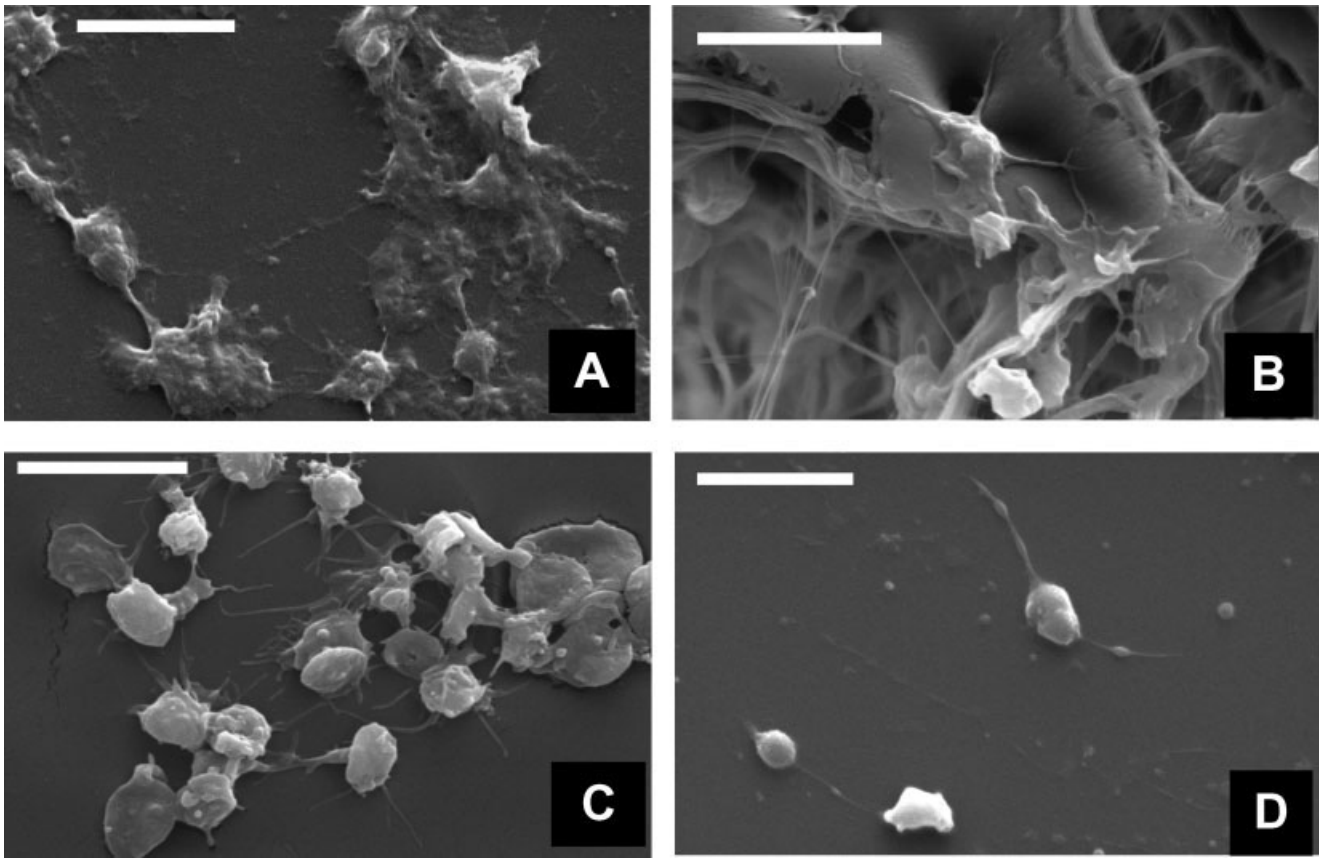

Figure 1. Representative SEM images of human platelet-rich plasma (PRP) incubated on glass (A), ePTFE (B), PLGA (C), and POC (D). There are many more adhered platelets on glass, ePTFE, and PLGA when compared with POC. Platelets on glass, ePTFE, and PLGA are spread, unlike those on POC, which are spherical. For all images, scale bar = 5 μm.

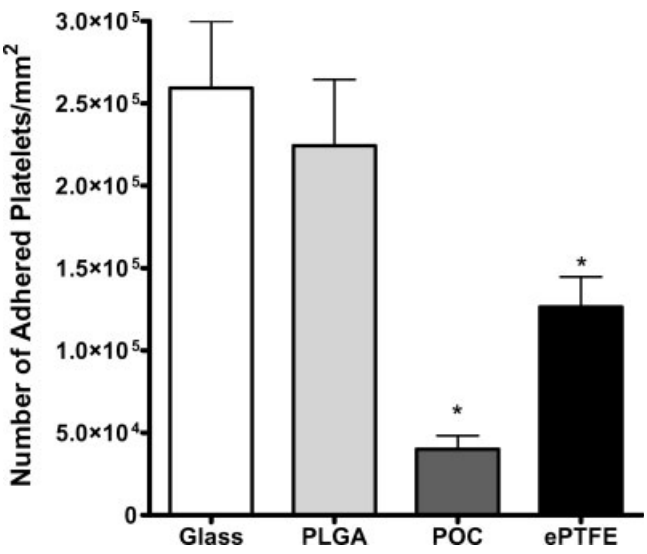

Figure 2. Quantification of platelet adhesion on glass, PLGA, POC, and ePTFE as determined by quantification of LDH activity. For each sample, platelet attachment was normalized by area (mm^2). *Corresponds to a $p < 0.05$ in comparison to platelet adhesion to glass. $N = 21 \pm$ SEM.

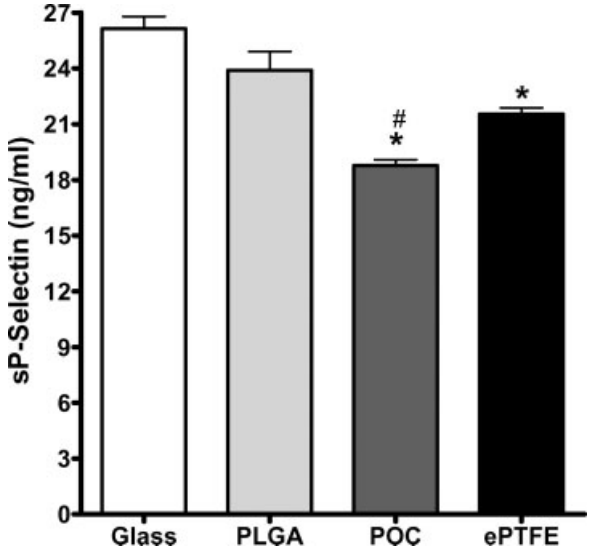

Figure 3. Determination of soluble P-selectin release in human plasma following incubation with glass, PLGA, POC, and ePTFE as determined by ELISA. *Corresponds to a $p < 0.01$, relative to glass. #Corresponds to a $p < 0.01$, relative to PLGA. $N = 6 \pm$ SEM.

Plasma recalcification profiles

The clotting process consists of both intrinsic and extrinsic pathways which ultimately lead to clot formation. The intrinsic cascade is initiated when contact is made between blood and surfaces.[27,28] The extrinsic pathway is initiated upon vascular injury which leads to exposure of tissue factor (TF) (also known as factor III).[27] Classical biochemistry of the intrinsic clotting pathway suggests that a "pro-coagulant stimulus," starting with exposure of plasma to a test surface, potentiates a cascade of events that culminate into the release of thrombin (Factor IIa).[29] Calcium is an activating factor that is involved in the progression of the intrinsic pathway of clot formation. The plasma recalcification profiles serve as a measure of the intrinsic coagulation system. Typical recalcification clotting profiles and clotting rates for POC, TCP, and PLGA are shown in Figure 4(A). The absorbance increases as the plasma becomes more turbid, correlating with the formation of a clot. A rightward shift of the curve indicates an overall slower clot time; whereas a leftward shift of the curve indicates a faster clot time. The initial slope of the linear region of the curve between inflection points is a measure of the rate of clot formation [Fig. 4(B)]. Citrated plasma (without the addition of $CaCl_2$) serves as a negative control, as it does not clot within the experimental time. TCP was the most coagulative surface as evidenced by a leftward shift in the curve, and the steepest slope relative to all other surfaces tested. Plasma samples on PLGA and POC produced a rightward shift in the curve and a significant decrease in the slope of the curve relative to clot formation on TCP ($p < 0.05$). However, the rate of clot formation on POC is significantly lower than on both, PLGA and TCP [Fig. 4(B)]. As mentioned in the methods section, because of the opaqueness of ePTFE, it was not included in this analysis. Overall, the rightward shift of the clotting profile on POC and the decreased slope suggest that POC is less coagulative than both TCP and PLGA.

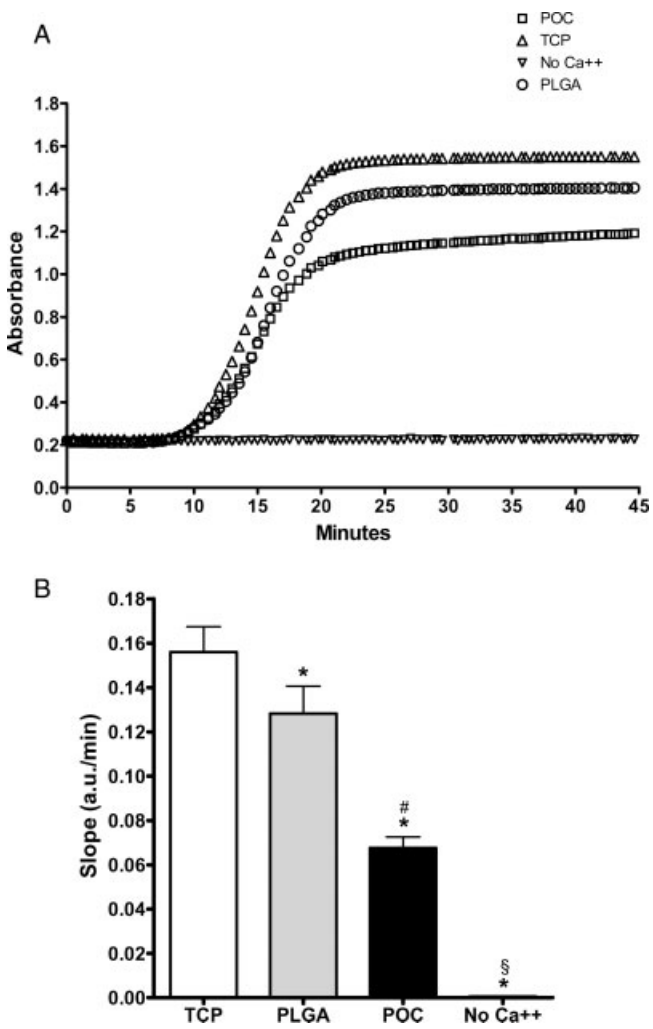

Figure 4. Plasma recalcification kinetic profiles of the absorbance at 405 nm as a function of time for PPP incubated with TCP, PLGA, and POC. Citrated PPP (without the addition of calcium) was used as a negative control (A). The data were averaged over six wells and are representative of three independent experiments. Clotting rate for each surface was calculated using the slope of the linear portion of the absorbance versus time data (B). *Corresponds to a $p < 0.05$ in comparison to TCP. #Corresponds to a $p < 0.001$ relative to PLGA. §Corresponds to a $p < 0.001$ relative to TCP, PLGA, and POC. $N = 6 \pm$ SEM.

In vitro assessment of inflammatory potential

Upon exposure of blood to a biomaterial, there is the possibility of generating an inflammatory response, which relies heavily on monocytes and macrophages. The chronic inflammatory response is mediated by monocytes; therefore, it is important to assess the effect of biomaterials on monocyte activation. Activated peripheral blood monocytes (PBM) release inflammatory markers, including IL-1β and TNF-α. Because of the challenges associated with using PBM; a suitable cell line has been developed that overcomes some of the obstacles.[30] For the analyses described herein, the human THP-1 cell line, which has been widely used as an alternative to PBM, was used because this cell line mimics the secretory response of PBM.[30,31] THP-1 cells incubated with 10 ng LPS controls demonstrated an increase in TNF-α and IL-1β generation. TNF-α secretion was significantly decreased in polystyrene (PS), PLGA, ePTFE, and POC relative to the LPS control ($p < 0.01$) [Fig. 5(a)]. THP-1 cells incubated with POC (7.94 ± 0.59 pg/mL), ePTFE (6.92 ± 1.3 pg/mL), PLGA (8.98 ± 1.2 pg/mL), and PS (6.84 ± 0.75 pg/mL) elicited very little response. Similarly, IL-1β secretion was significantly reduced in PS (2.37 ± 0.21 pg/mL), PLGA

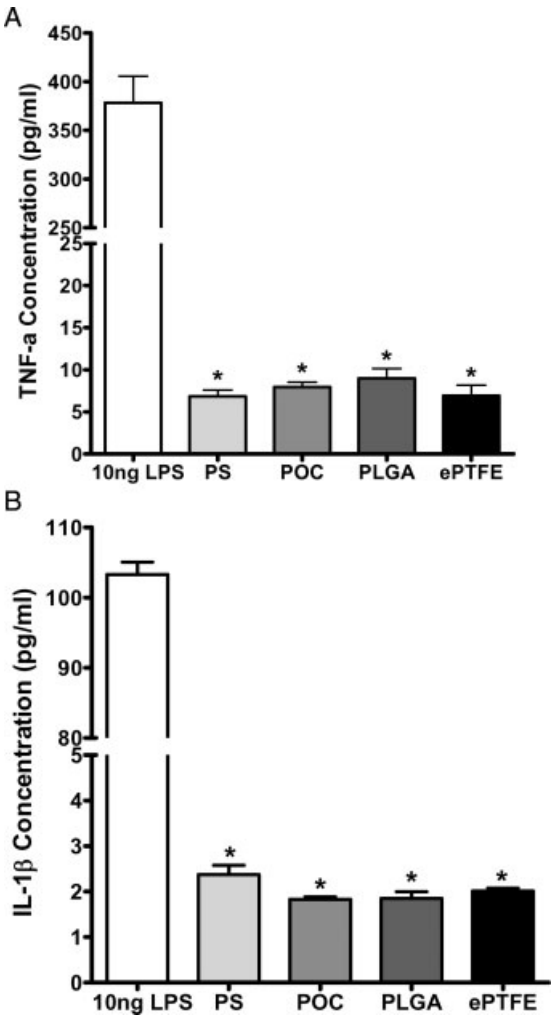

Figure 5. The quantification of TNF-α (A) and IL-1β (B) released from THP-1 cells following incubation with TCP, POC, PLGA, and ePTFE as determined by ELISA. LPS-stimulated (10 ng) THP-1 cells served as positive controls. *Corresponds to a $p < 0.01$ in comparison to LPS stimulated control. $N = 5 \pm$ SEM.

(1.85 ± 0.15 pg/mL), ePTFE (2.01 ± 0.06 pg/mL), and POC (1.83 ± 0.05 pg/mL) samples when compared with LPS the control ($p < 0.01$) [Fig. 5(B)]. The minimal generation and release of immunoreactive cytokines (TNF-α and IL-1β) from THP-1 cells upon incubation with POC, ePTFE, and PLGA supports the *in vitro* hemocompatibility of these materials and confirms *in vivo* biocompatibility data in pigs.[6]

Hemolysis

The hemolytic activity of biomaterials plays an important role with regards to toxicity and correlates with inhibition of cell growth.[32] The polymers tested, including POC, show no evidence of hemolysis after 2 h of incubation (<0.5% hemolysis) (Fig. 6). These data confirm that like the two other synthetic materials tested, ePTFE and PLGA, POC does not promote hemolysis.

Protein adsorption

Protein adsorption onto a surface is the first step when blood comes in contact with a surface. Adsorbed fibrinogen can mediate platelet and endothelial cell adhesion to differing extents depending on the conformation of the adsorbed protein.[33] In this study we focused on quantification of total adsorbed fibrinogen as an initial indicator of the surface's "adhesiveness" to proteins. Nitrocellulose had the highest level of fibrinogen adsorption relative to all other materials (25 ± 3 μg/mm^2 ($p < 0.001$)) (Fig. 7). There were statistically significant differences in protein adsorption among the other test surfaces with the low protein binding surface exhibiting the least amount of adsorption as expected (POC (1.2 ± 0.3 μg/mm^2), PLGA (1.0 ± 0.2 μg/mm^2), ePTFE (0.9 ± 0.3 μg/mm^2), and the low protein binding plastic (0.7 ± 0.2 μg/mm^2)). Although there were slight differences in the mean value of fibrinogen adsorbed to POC, ePTFE, and PLGA, differences in platelet or cell adhesion could be caused by the presence of other proteins in media (or blood) and the conformation of adsorbed fibrinogen.[33]

Cell differentiation and resistance to shear stress

Immunohistochemistry data demonstrate that HAECs cultured on POC stain positive for EC-specific markers VE-cadherin and vWF [Fig. 8(A,B)]. The staining of HAECs for VE-cadherin shows the characteristic localization within the cytoplasm and at the

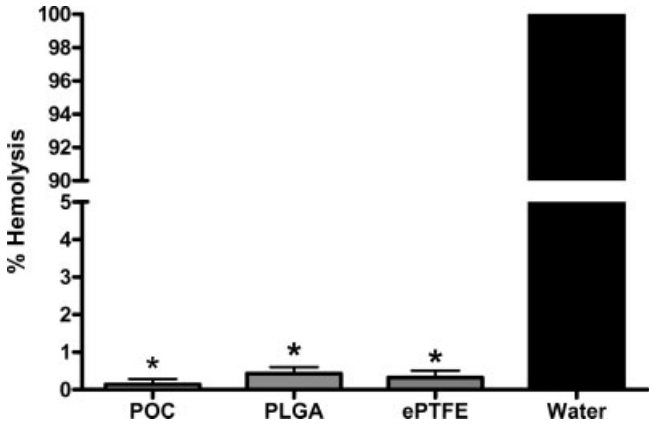

Figure 6. Hemolysis of red blood cells following incubation of whole blood with POC, PLGA, and ePTFE. Blood samples exposed to complete hemolysis with water were used to normalize the data. *Corresponds to a $p < 0.001$ in comparison to control. N = 15 ± SEM.

cell periphery. Staining of vWF is also characteristically localized to granules in the cytoplasm. Both of these stains confirm that the HAECs cultured on POC maintain the endothelial cell phenotype. Regarding cell attachment under flow conditions, there was a 69% ± 7% and 102% ± 8% cell retention on POC and glass, respectively ($p < 0.05$) [Fig. 9(A)]. Representative phase contrast images show qualitatively the relative number of HAECs still attached to POC and glass surfaces after exposure to fluid shear stress [Fig. 9(B–D)]. The mean arterial hemodynamic shear stress lies between 15 and 20 dyn/cm^2.[34,35] More than half the HAECs remained attached to POC following exposure to physiologic shear stress. Despite the significant difference in HAEC retention between glass and POC surfaces, the percent retention of HAECs on POC after exposure to fluid shear remains promising. The POC and glass surfaces were not treated or modified in any way to enhance HAEC attachment and spreading. Cellular attachment was only facilitated by the POC and glass surface properties and nonspecific adsorption of serum proteins already present in the cell culture media. Future work can be done to increase cell retention to POC by utilizing methods such as surface coating with fibronectin or collagen, immobilization of adhesion peptides, and shear stress preconditioning of cultured cells. Similar cell attachment work using human umbilical vein endothelial cells has been investigated using collagen-treated PTFE, Dacron (PET), and polyurethane (PU) surfaces.[36] In the studies, the percent cell retention on PTFE, PET, and PU surfaces after a 1-h exposure to a shear stress of 20 dyn/cm^2 was 49% ± 20%, 30% ± 2% and 40% ± 14% (mean ± SD), respectively. These numbers are below the 69% ± 7% (mean ± SEM) HAEC retention on POC at a similar shear stress value.

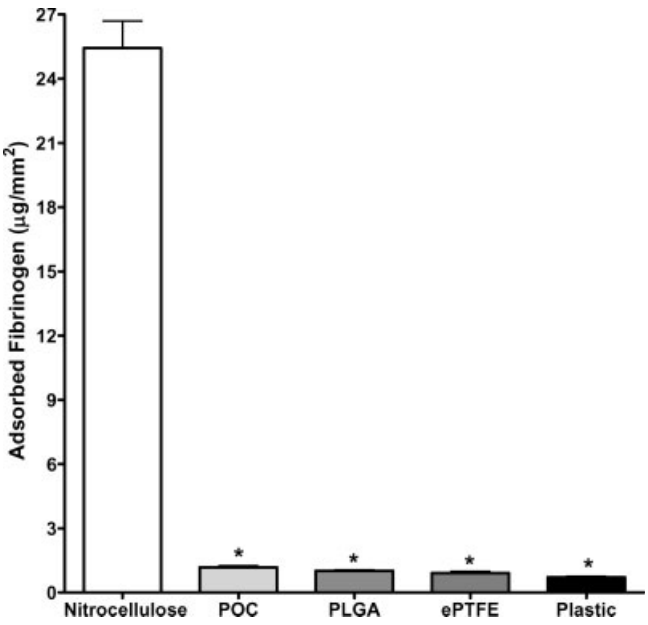

Figure 7. Analysis of fibrinogen protein adsorption to POC, PLGA, ePTFE, and untreated plastic. Nitrocellulose membranes serve as positive control surface for maximal fibrinogen absorption. *Corresponds to a $p < 0.001$ in comparison with nitrocellulose control. $N = 5 \pm$ SEM.

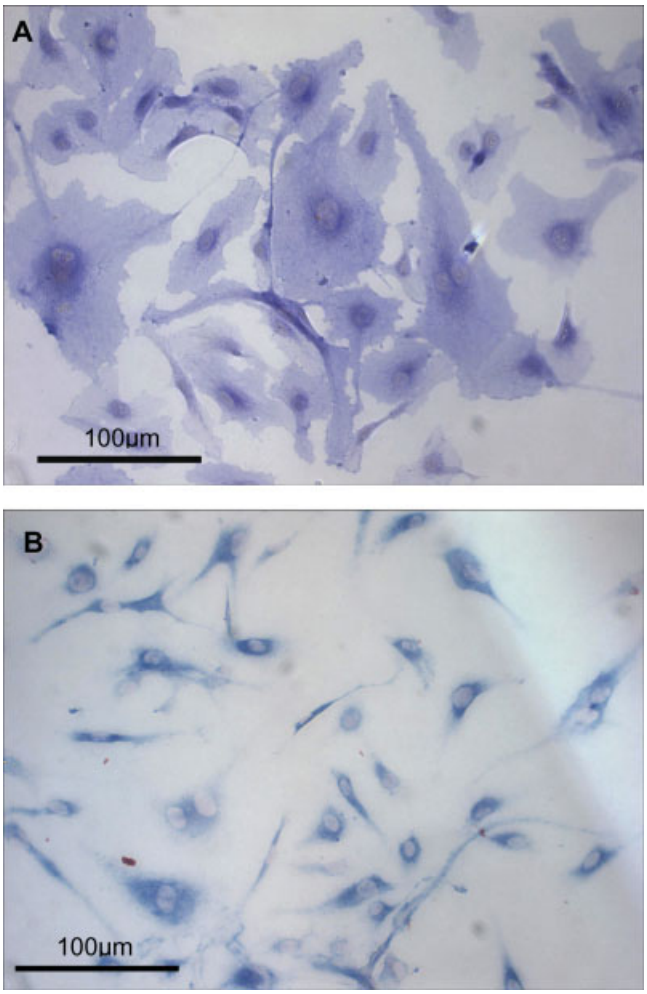

Figure 8. Human aortic endothelial cells cultured on POC stain positive for endothelial cell-specific markers, VE-cadherin (A) and vWF (B). Images: ×200. For both images the scale bar is 100 µm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CONCLUSION

The hemocompatibility of POC was evaluated *in vitro* to determine its utility as a scaffold for *in vivo* vascular tissue engineering. The platelet adhesion, clotting, inflammatory potential, hemolysis, and protein adsorption data suggest that POC is hemocompatible. In addition, the endothelial cell attachment data were encouraging and suggest that with further

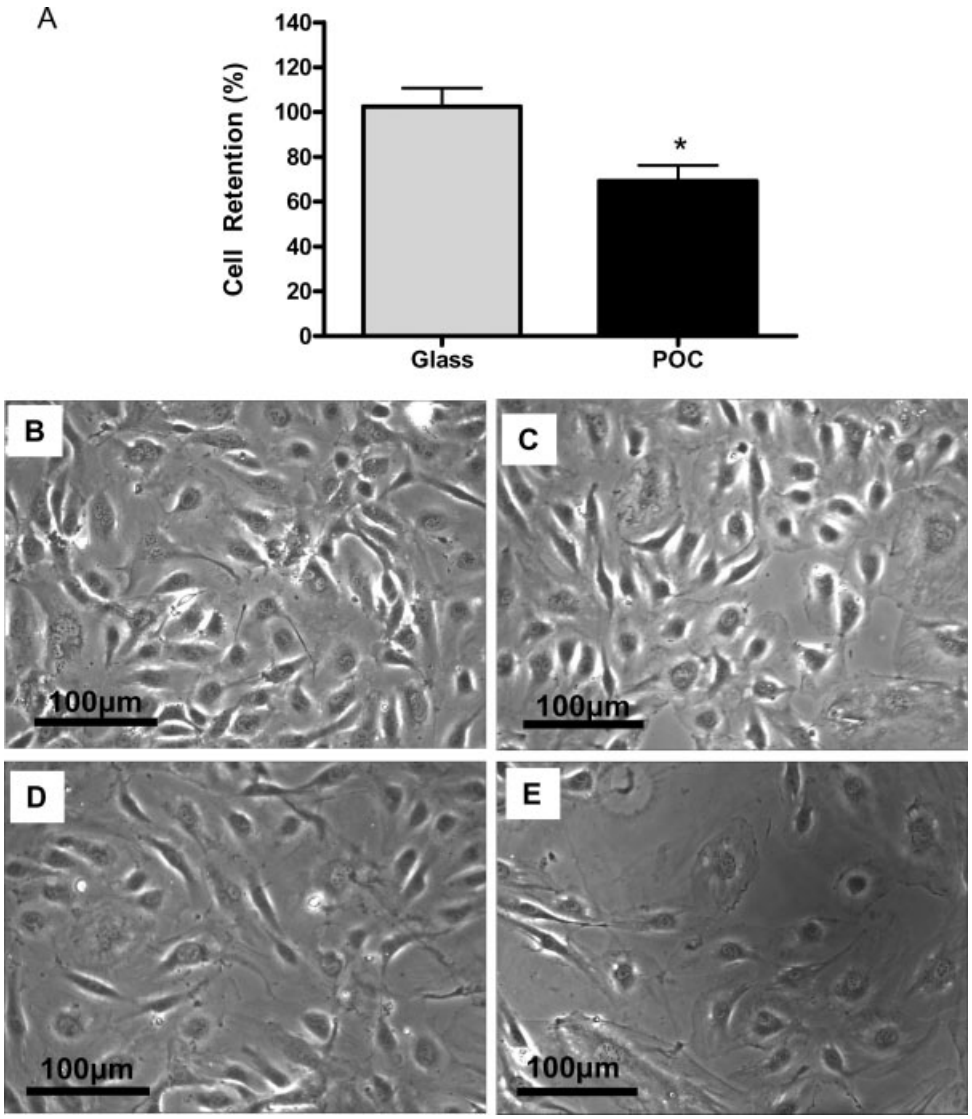

Figure 9. Percent HAEC retention on glass and POC surfaces after a 1-h exposure to a shear stress of 20 dyn/cm^2 (A). HAEC cell retention is taken as a percentage relative to the number of cells attached under static conditions. (Glass $n = 5$, POC $n = 6$, data are mean $\pm$ SEM). *Corresponds to a $p < 0.05$ relative to glass. Representative phase contrast images of HAECs attached to glass or POC under static culture and after a 1-h exposure to fluid shear stress of 20 dyn/cm^2. Static culture glass (B), flow glass (C), static culture POC (D), and flow POC (E).

modification, POC vascular scaffolds should function under physiological flow conditions.

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