Hemocompatibility evaluation of poly(glycerol-sebacate) in vitro for vascular tissue engineering

Delara Motlagh, Jian Yang, Karen Y. Lui, Antonio R. Webb, Guillermo A. Ameer*

Biomedical Engineering Department, Northwestern University, 2145 Sheridan Road, Room E310, Evanston, IL 60208, USA

Received 8 February 2006; accepted 1 April 2006

Abstract

Poly(glycerol-sebacate) (PGS) is an elastomeric biodegradable polyester that could potentially be used to engineer blood vessels in vivo. However, its blood-material interactions are unknown. The objectives of this study were to: (a) fabricate PGS-based biphasic tubular scaffolds and (b) assess the blood compatibility of PGS in vitro in order to get some insight into its potential use in vivo. PGS was incorporated into biphasic scaffolds by dip-coating glass rods with PGS pre-polymer. The thrombogenicity (platelet adhesion and aggregation) and inflammatory potential (IL-1β and TNFα expression) of PGS were evaluated using fresh human blood and a human monocyte cell line (THP-1). The activation of the clotting system was assessed via measurement of tissue factor expression on THP-1 cells, plasma recalification times, and whole blood clotting times. Glass, tissue culture plastic (TCP), poly(l-lactide-co-glycolide) (PLGA), and expanded polytetrafluoroethylene (ePTFE) were used as reference materials. Biphasic scaffolds with PGS as the blood-contacting surface were successfully fabricated. Relative to glass (100%), platelet attachment on ePTFE, PLGA and PGS was 61%, 100%, and 28%, respectively. PGS elicited a significantly lower release of IL-1β and TNFα from THP-1 cells than ePTFE and PLGA. Similarly, relative to all reference materials, tissue factor expression by THP-1 cells was decreased when exposed to PGS. Plasma recalification and whole blood clotting profiles of PGS were comparable to or better than those of the reference polymers tested.

Keywords: Hemocompatibility; Blood compatibility; Coagulation; Thrombosis; Vascular grafts; Tissue engineering

1. Introduction

It is estimated that 1.2 million Americans will suffer a heart attack this year, resulting in nearly 500,000 deaths, of which 50% are due to coronary heart disease [1]. In treating this disease, approximately 500,000 bypasses are performed every year with autologous blood vessels as the first option for revascularization. Nevertheless, up to 30% of the patients who require arterial bypass surgery do not possess suitable or sufficient autologous blood vessels, necessitating the use of synthetic grafts [2]. Polyethylene-terephthalate (PET) and expanded polytetrafluoroethylene (ePTFE) are currently the standard biomaterials used for prosthetic vascular grafts [3]. Satisfactory results have been obtained with PET or ePTFE vascular grafts used to replace or bypass, large-diameter blood vessels; however, results reported when used in small-diameter blood vessels (< 6 mm) are poor when compared to those of autologous vein grafts [4]. For example, when used for femoropopliteal bypass grafting, PET and ePTFE are only 36% and 47% functional after two years, respectively [5].

To address this problem, small-diameter vascular grafts have become a major area of interest, spanning multiple disciplines. A variety of biodegradable polymers, scaffolds, and matrices have been evaluated for the development of a tissue engineered vascular graft [6,7]. The tissue engineering approach would rely on either the in vitro or in vivo cellular remodeling of a polymeric scaffold. In order for in vivo cellular remodeling to be successful, the biocompatibility, degradation rate and mechanical characteristics of the scaffold must be well-suited to the dynamic environment of the blood vessel. The ideal scaffold for vascular tissue engineering should employ a biocompatible and degradable polymer with elastomeric properties that interact favorably with cells and blood.
Poly(glycerol-co-sebacate) (PGS) is an elastomeric biodegradable polymer developed recently for use in soft tissue engineering [8,9]. It has been investigated for potential uses in regenerative medical approaches including the development of an artificial microvasculature [10] and a nerve guidance material [11]. Also, due to the elastomeric and degradation properties of PGS, it could potentially be used to engineer blood vessels in vivo. However, the incorporation of PGS into a scaffold for vascular tissue engineering and the hemocompatibility of this material have not been investigated. In this study, the fabrication of PGS-based biphasic tubular scaffold and the interaction of PGS in vitro with blood and plasma were investigated in order to assess its application in vascular tissue engineering.

2. Experimental

2.1. Buffers and cells

The phosphate-buffered saline (PBS) contained 137 mM NaCl, 2.7 mM KCl, 10.1 mM sodium phosphate dibasic and 1.8 mM potassium phosphate monobasic, pH = 7.4. The platelet-suspending buffer (PSB) contained 137 mM NaCl, 2.7 mM KCl, 0.4 mM Sodium Phosphate Monobasic, 5.5 mM Dextrose, 10 mM HEPE, 2.5 mM CaCl₂, 0.1 U/mL Apyrase, 1.0 mM MgCl₂ and 4 mg/mL BSA, pH = 7.4. The Triton-PSB buffer contained 2% (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PSB, pH = 7.4. The glutaraldehyde buffer contained 2.5% (v/v) glutaraldehyde in PBS, pH = 7.4. One milliliter of 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 mM HEPE, 1.0 mg sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10% newborn calf serum, and 0.05 mM 2-mercaptoethanol). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

2.2. Preparation of samples

Equimolar amounts of glycerol and sebacic acid were added to a 250 ml three-neck round-bottom flask fitted with an inlet and outlet adapter. Sebacic acid and glycerol were added in a 1:1 molar ratio and melted at 150 °C under a flow of nitrogen gas while stirring to create a prepolymer. For the fabrication of flat sheets, the prepolymer was stirred for an additional 4 h under a vacuum of 50 mmHg and then cast into a glass Petri dish pre-heated to 120 °C and immediately placed into a 120 °C vacuum oven. The co-polymer was kept at 0.015 mmHg and 120 °C vacuum for an additional 8 h under 50 mmHg vacuum to obtain a PGS sheet. For biphasic scaffold fabrication, the prepolymer was mixed at 120 °C for an additional 8 h under 50 mmHg vacuum to create a viscous solution that would not phase separate once removed from the heat. To test the feasibility of incorporating PGS into a hybrid biphasic scaffold design, PGS solid tubes (inner layer) were first fabricated using a solvent coating and evaporation technique. Brieﬂy, PGS prepolymer was dissolved in acetone to a concentration of 35% (w/v). Glass rods were transferred to a vacuum oven for post-polymerization at 120 °C for 72 h under 0.015 mmHg. A porous poly(1,8-octanediol citrate) (POC) [12,13] (outer layer) was added to the PGS tube by placing the PGS-coated glass rod concentrically into a Teflon tube mold that contained a salt-POC prepolymer slurry with 90% salt particles (100–200 μm). The assembly was then post-polymerized at 80 °C for 4 days. This method for the fabrication of biphasic scaffolds has been previously described [13,14]. Expanded PTFE samples were donated by the Division of Vascular Surgery, Feinberg Medical School, Northwestern University. A transparent PLGA 85/15 (Sigma-Aldrich, St. Louis, MO) film was prepared by dissolving Poly(lactide-co-glycolide) (PLGA) 85/15 in dichloromethane, which was cast into a poly(tetrafluoroethylene) mold. The solvent was evaporated at room temperature, producing the film. The PLGA 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. All samples were cut into 10 mm diameter disks using a cork borer with a surface area of 78.5 mm².

2.3. 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 on Research Subjects at Northwestern University. Platelet-rich plasma (PRP) was prepared as previously described [15]. Briefly, whole blood was centrifuged at 250g 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, #732-1010) were packed with sepharose 2B (Sigma-Aldrich, St. Louis, MO, #2B-300) and equilibrated with PSB. The PRP was run through the column and the elution volumes containing the platelets were collected and the platelet concentration determined prior to incubation with samples.

2.4. Quantification of platelet adhesion

Glass, PGS, PLGA, and ePTFE samples were incubated with the PRP for 1 h at 37 °C under static conditions. The suspension was aspirated and ePTFE samples 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 [15,16]. 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, 1644793) was added and incubated for 20 min at 37 °C. The reaction was stopped with the addition of 1 N HCl. The optical density was measured at 490 nm with 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).

2.5. Quantification of platelet activation via detection of soluble P-selectin (sP-selectin)

Glass coverslips, PLGA, PGS 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 added to a final concentration of 10 mM. The sample was subsequently centrifuged at 2000g for 10 min to obtain the platelet poor plasma (PPP) [17]. The concentration of sP-selectin levels in the plasma was determined using ELISA kit (Human soluble P-selectin Immunassy, R&D Systems, Minneapolis, MN, #BBE 6).

2.6. Quantification of whole blood clotting time

The thrombogenicity of PGS was evaluated using a whole blood kinetic clotting time method, as previously described [18,19]. Blood was drawn from healthy adult volunteers by venipuncture into ACD anticoagulant vacutainer tubes. The first 3 ml of blood drawn was discarded to prevent contamination by tissue thromboplastin caused by needle puncture. Four
samples, each 10 mm in diameter, were used per time point. Briefly, the clotting reaction was activated with the addition of 850 μL CaCl₂ (0.1 M) to the 8.5 mL sample of ACD blood. A 100 μL volume of the activated blood was carefully added to glass coverslips, PLGA, PGS and ePTFE samples, which were placed in the wells of a 12-well plate. All samples were incubated at room temperature for 5, 15, 25, 35 and 45 min. At the end of each time point, the samples were incubated with 3 mL of distilled water for 5 min. Each well was sampled in triplicate (200 μL each) and transferred to a 96-well plate. The red blood cells that were not trapped in a thrombus were lysed with the addition of distilled water, thereby releasing hemoglobin into the water for subsequent measurement. The concentration of hemoglobin in solution was assessed by measuring the absorbance at 540 nm using a 96 well plate reader. The size of the clot is inversely proportional to the absorbance value.

### 2.7. Measurement of plasma recalcification profiles

Blood was drawn from healthy adult volunteers in to ACD tubes (as described above) and spun at 2000 g in order to obtain the PPP. PLGA and PGS 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 CaCl₂. Following the addition of PPP, 100 μL of 0.025 M CaCl₂ was added to each well (except the no Ca⁺², negative control). The plate was then immediately placed in a 96 well plate reader, where the kinetics of the clotting process due to recalcification 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, six 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. Due to the opaque nature of ePTFE, it was not tested with this method.

### 2.8. Measurement of TNF-α, IL-1β release and tissue factor expression

Each vial of THP-1 cells (human acute monocytic leukemia cell line) was thawed and grown in suspension in 5 mL of complete RPMI-1640 media for several days at 37 °C, 5% CO₂. The cells were collected and concentrated (10⁶ cells/mL), then 1 mL was placed in each well of a 48-well tissue culture plate containing PLGA, PGS, ePTFE, TCP and Lipopolysaccharide (LPS, 10 ng/mL and 10 μg/mL). THP-1 cells were incubated with the samples at 37 °C for 18 h under static conditions. Cells were pelleted and the supernatant removed. The concentrations of TNF-α and IL-1β in the supernatants were determined by sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). The cell pellet was resuspended in 100 μL of 1% Triton X-100 in Tris Buffered Saline (TBS, pH 8.5) and incubated at 4 °C for 1 h. The IMUBIND Tissue Factor ELISA kit (American Diagnostica Inc., Stamford, CT) was used to measure the amount of tissue factor in the pellet fraction, as per the manufacturer’s instructions.

### 2.9. Statistical analysis

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

### 3. Results and discussion

#### 3.1. Synthesis of PGS scaffolds

Current synthetic materials used to bypass arteries less than 6 mm in diameter have thrombosis rates of at least 40% after 6 months [20]. As a functional endothelium can control thrombus formation, lining the lumen of vascular grafts with autologous endothelial cells prior to implantation has been investigated as a means to improve the efficacy of small-diameter vascular grafts [21]. It has been previously reported that PGS supports endothelial cell attachment [8,22], making it a suitable candidate material for the lumen of an engineered blood vessel.

We have previously reported the design and fabrication of a biphasic scaffold for vascular tissue engineering [12,13]. Fig. 1a shows a biphasic scaffold featuring a solid PGS lumen and a porous POC outer layer. The biphasic micro-architecture of the scaffold is seen in greater detail in Fig. 1b. One benefit of this biphasic design is that it provides a three-dimensional, outer porous layer onto which smooth muscle cells can be seeded. The interconnected porosity of this layer will facilitate the migration and proliferation of cells. The incorporation of PGS as an inner nonporous layer of the scaffold promoted the investigation of its hemocompatibility characteristics. The inner lumen is expected to serve as a surface for in vitro or in vivo endothelialization. The surface topography and transport properties of this layer can be modified, independent of the outer porous layer, if necessary.

![Fig. 1. A biphasic scaffold featuring a solid PGS lumen and a porous POC outer layer is seen in (a). The biphasic nature of the scaffold is seen in greater detail in (b).](image)
3.2. Platelet adhesion and activation

Platelet spreading and aggregation are markers of platelet activation [23] and are thought to be a major mechanism by which biomaterial thrombogenicity is transduced; therefore, in vitro blood compatibility tests have typically involved measurement of platelet adhesion and granule release [24,25]. Platelets that adhered to glass displayed a drastic change in their morphology (Fig. 2a and w). Platelets that attached and aggregated on ePTFE displayed similar morphological alterations as those attached on glass, though to a lesser extent (Fig. 2c and y). Fewer platelets adhered to PGS and most maintained a rounded morphology (Fig. 2b and x), suggesting that they were less activated. In contrast, platelets that adhered to PLGA appear to aggregate and spread on the surface (Fig. 2d and z). The number of platelets that adhered to PLGA was significantly lower than that of the other polymers considered here due to its negatively charged surface, which could serve as a reliable marker of platelet activation [30–33], and would theoretically take into account activation of different blood components when evaluating the hemocompatibility of the biomaterial. These data suggest that under the conditions tested, ePTFE is less thrombogenic than PGS and PLGA (p<0.05). Nevertheless, PGS and PLGA are less thrombogenic than glass.

3.3. Whole blood clotting time

There are many potential reasons why grafts fail, but the most prevalent problem is occlusion due to blood coagulation, particularly under conditions of relatively low flow [6]. Whole blood was used to assess clotting times. In this assay, higher absorbance values correlate with improved thromboresistance of the material (Fig. 5). Blood incubated with glass completely clotted at 45 min and therefore used as a reference thrombogenic material. At each time point measured, blood incubated with glass had a significantly lower absorbance than PLGA, PGS and ePTFE (p<0.05, n=10), indicating that it clotted more rapidly. There was no significant difference in the degree of clotting between PLGA, PGS and ePTFE until 35 min, at which point blood incubated with the PGS had a higher degree of clotting than ePTFE (p<0.05, n=7); whereas, no difference was observed between PLGA and ePTFE at 35 min (p>0.05, n=7). The absorbance for PGS, ePTFE, PLGA, and glass was 0.536±0.084, 0.903±0.033, 0.651±0.082, and 0.043±0.016, respectively, at 45 min. Despite a reduced platelet attachment to PGS relative to ePTFE, the blood clotted more rapidly upon incubation with PGS. This finding underscores the importance of using various assays that assess the contributions of different blood components when evaluating the hemocompatibility of the biomaterial. These data suggest that under the conditions tested, ePTFE is less thrombogenic than PGS and PLGA (p<0.05). Nevertheless, PGS and PLGA are less thrombogenic than glass.

3.4. Plasma recalcification profiles

Coagulation is the culmination of a series of reactions, ultimately resulting in the thrombin-catalyzed transformation of fibrinogen into an insoluble fibrin clot. Thrombin is formed upon the convergence of the intrinsic and extrinsic pathways of coagulation. Viewed from the perspective of blood-biomaterial interactions, the intrinsic pathway is triggered by surface contact (particularly surfaces with a negative charge) [34] and employs a linear cascade of reactions, requiring Ca2+. Plasma recalcification profiles serve as a measure of the intrinsic coagulation system. The absorbance increases as the plasma becomes more turbid, correlating with the formation of a clot. A rightward shift of the curve indicates a slower clotting time; whereas, a leftward shift of the curve indicates a faster one. Citrated platelet poor plasma (without the addition of CaCl2) serves as a negative control, as it should not form a clot. Plasma incubated with TCP and PLGA produced a leftward shift in the kinetic profiles; whereas, PGS caused a rightward
shift in the curve, indicating a longer clotting time (Fig. 6a). The initial slope of the linear portion of each profile (Fig. 6b) was calculated as a measure of the rate of clot formation. No difference was observed between the slopes of TCP and PLGA ($p > 0.05$), but both were significantly higher than PGS ($p < 0.05, n = 6$), indicating an increase in

Fig. 2. SEM images of human platelet-rich plasma (PRP) incubated on glass (a), PGS (b), ePTFE (c) and PLGA (d). On the right, panels w, x, y and z are magnified photographs of a, b, c and d, respectively. There are many more adhered platelets on glass, ePTFE and PLGA as compared to PGS. Platelets on glass, ePTFE and PLGA are spread, unlike those on PGS which are spherical.
the rate of clotting on these surfaces via the intrinsic coagulation pathway. The clotting time to reach half-maximal absorbance (half-max time) was measured for each surface (Fig. 6c). The half-max time for PGS (16.48 min ± 0.6754) was significantly higher than that of PLGA (13.0 ± 0.705 min, n = 6) or TCP (13.8 ± 0.214 min, p < 0.01), indicating slower clotting on PGS. Taken together, the right shift of the kinetic profile, the decrease in the slope (Fig. 6b) and the increased half-maximal absorbance clotting time (Fig. 6c) suggest that PGS is less coagulative than both TCP and PLGA. As mentioned in the Methods section, ePTFE samples were not evaluated as its opaque nature made it unsuitable for this assay.

3.5. In vitro assessment of the activation of the extrinsic coagulation pathway

Tissue factor expression can indicate the level of activation via the extrinsic coagulation pathway. THP-1 cells incubated with the positive control (10 μg LPS) expressed high amounts of tissue factor (942 pg/ml ± 16.9) (Fig. 7); whereas, significantly lower amounts were expressed by cells exposed to TCP, PLGA, ePTFE or PGS (p < 0.001, n = 4). Exposure of cells to PGS resulted in significantly lower expression of tissue factor (136 pg/ml ± 3.99, n = 5) than when cells were exposed to TCP (502 pg/ml ± 5.24), PLGA (651 pg/ml ± 19.6) or ePTFE (577 pg/ml ± 51.9, p < 0.001). This finding suggests less potential for extrinsic pathway activation and coagulation by the PGS surfaces.
It is important to note that the intrinsic and extrinsic pathways do not work completely independent from one another. In fact, it has been suggested that the extrinsic pathway predominantly initiates coagulation, while the intrinsic pathway is the major force behind the propagation phase [35]. More research in this area is needed in order to assess the relative impact of each pathway in the course of coagulation in the presence of biodegradable biomaterials.

3.6 In vitro assessment of inflammatory potential

Monocytes comprise 5% of circulating leukocytes in the blood and have been shown to be critical in the biological response to biomaterials [36] in that they direct much of the inflammatory response. Activated peripheral blood monocytes (PBM) release inflammatory markers, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). In fact, monocytes have previously been used to study biomaterials [36–39]. There are disadvantages to using PBMs in that they introduce variables (such as sex, age and health) depending on donor population. In addition, preparations may become contaminated with other leukocytes. PBMs are problematic in long-term cultures and are difficult to normalize against standards in assessing biological significance, limiting their use for materials screening [40]. The human THP-1 monocyte cell line mimics many of the secretory functions of PBMs [41,42] and has been widely used as an alternative to peripheral blood monocytes [40–45]. The biological response of PBMs and THP-1 cells to biomaterials have been

![Clotting kinetic profiles](image-url)
compared and reported to be equivalent [40,41], justifying the use of THP-1 cells as a model for biomaterial evaluation.

TNF-α release (Fig. 8a) was significantly decreased in TCP, PLGA, ePTFE and PGS as compared to LPS controls (p < 0.001, n = 4); moreover, cells incubated with PGS contained less TNF-α (10.2 pg/ml ± 0.85, n = 5) than ePTFE samples (25.1 pg/ml ± 2.57, p < 0.01). Similarly, IL-1β release (Fig. 8b) was significantly reduced in TCP, PLGA, ePTFE and PGS samples (p < 0.001, n = 4) as compared to LPS controls. The minimal generation of immunoreactive cytokines (TNF-α and IL-1β) from THP-1 cells exposed to PLGA, ePTFE and PGS reiterates the biocompatibility of these materials. Although TNF-α and IL-1β mediate and amplify inflammation, they are produced by different pathways. This convergence of signals could serve as a mechanism by which to further regulate this critical process.

4. Conclusions

PGS was incorporated into a scaffold for vascular tissue engineering and its hemocompatibility in vitro relative to ePTFE, PLGA, TCP, and glass was evaluated. A biphasic
scaffold was fabricated with PGS in the blood-contacting surface. Taken together, the platelet adhesion, clotting and inflammatory potential data suggest that PGS is a hemocompatible material warranting further investigation for use in vascular tissue engineering. Towards this end, it will be necessary to examine other factors such as smooth muscle cell proliferation, gene expression and extracellular matrix production.

Acknowledgements

This research was supported by grants from Baxter/IBNAM (The Institute for BioNanotechnology in Medicine) to Dr. Motlagh (The Early Career Development Award) and the National Institutes of Health grant #R21HL071921 to Dr. Ameer.

References


