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Original Article

In vitro hemocompatibility evaluation of poly (4-hydroxybutyrate) scaffold

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Abstract: The biosynthetic thermoplastic poly-4-hydroxybutyrate (P4HB) possesses favorable tensile strength and elongation performance and has been studied as a new implantable polymer material for medical uses. However, its hemocompatibility has not been tested to confirm its applicability to tissue engineering. In this study, a series of experiments was performed that included erythrocyte hemolysis tests, dynamic blood coagulation tests, platelet adhesion tests, effects on blood coagulation, Wright staining, and adsorption of erythrocytes, leukocytes, platelets, and plasma proteins. The results were compared with control tests on polyvinyl chloride (PVC), a biomaterial in current use, to evaluate the relative in vitro hemocompatibility of P4HB. The degree of hemolysis in the presence of P4HB was 1.9 ± 0.2%. The absorbance-time curve for blood clotting declined slowly and smoothly. There were no differences in the test values of Factor XII, activated partial thromboplastin time (APTT), or fibrin degradation products (FDPs) among whole blood samples exposed to P4HB or PVC and the blank control groups ($P > .05$). Adsorption of platelets and globulin was similar in samples exposed to P4HB and PVC ($P > .05$), but the adsorption of erythrocytes, leukocytes, and albumin to P4HB was higher ($P < .05$). In conclusion, P4HB compares favorably with PVC in terms of blood compatibility, except for a higher affinity for erythrocytes and leukocytes. The findings indicate that P4HB, an alternate scaffolding material with advantageous properties, is generally acceptable for bioengineering use.

Keywords: Biomaterial, poly-4-hydroxybutyrate, hemocompatibility, tissue engineering, scaffold

Introduction

Tissue engineering and regenerative medicine, which aim at the development of biological substitutes that restore, maintain, or improve organ function, have become popular research areas in life science during the last decade [1]. The rapid development of tissue engineering has promoted progress and evolution in the biomaterial and cell biology fields in regenerative medicine. However, functional tissue engineering applications remain rare in clinical usage. An important reason for this lack is the absence of suitable scaffold materials. Currently available scaffold materials tend to promote chronic inflammation, immune rejection, and thrombosis and to display poor mechanical properties that have limited their clinical applications [2].

As outlined in previous studies, an ideal scaffold should mimic the native microenvironment, which provides appropriate mechanical and biological properties for cell attachment, proliferation, and differentiation, and subsequently supports neo-tissue generation [3]. The materials also should be non-immunogenic and should be bioabsorbable in the host body over time, eventually leaving only the regenerated neo-tissue. Diverse materials have been developed and tested as scaffolds. At present, the scaffold materials can be grouped into 2 types: enucleated natural materials and biodegradable synthetic materials [4]. Some research has concluded that enucleated natural scaffolds are prone to variation, may transmit animal-based infectious diseases, and may provoke specific immune responses, as well as being quite expensive [5, 6]. Therefore, biodegrad-

able synthetic polymer scaffolds have been developed in recent years. The source, chemical composition, mechanical properties, and biological function of materials are all important attributes of tissue engineering biomaterials, especially for cardiovascular tissue engineering. Once implanted in the body, artificial cardiovascular tissue is in a complex hemodynamic environment, necessitating that the scaffolds have good hemocompatibility, elastomeric, and anti-fatigue properties [7]. Regardless of the nature of the scaffold material, once these exogenous materials are implanted, a series of complex reactions becomes possible if the materials contact blood directly, with ensuing potentially deleterious effects on clinical outcomes [8, 9]. Therefore, it is necessary to appraise the compatibility between the tissue engineering scaffold and blood.

The hemocompatibility of biomaterials refers to the degree of mutual adaptation between the materials and blood. This property arises from the interactions between each component of blood and the surface character of biomaterials, as well as the consequences of and effects produced by interactions. Because of the complex reaction mechanisms, various influencing factors, and variable internal environment in vivo, it is not possible to evaluate blood compatibility exhaustively by any single standardized method [10]. It is believed that hemocompatibility experiments in vitro and in vivo should include effects on blood coagulation, platelets, thrombosis, immunology, and other changes of hematology caused by biomaterials [11, 12].

Poly-4-hydroxybutyrate (P4HB) is a thermoplastic polymer that possesses remarkable tensile strength and elongation performance, while retaining flexibility and pliability. Unlike many such biosynthetic products, it is water insoluble and resistant to hydrolysis. Therefore, it is expected to be relatively stable upon exposure to the physiological environment. As a biosynthetic- (rather than a petrochemical-) derived product, it displays the important property of biodegradability. This makes it suitable for applications in which gradual replacement of the scaffold by tissue components is desired. P4HB is amenable to techniques currently used in the processing of plastics. Its major drawback may be the expense of biosynthetic production, a problem currently being addressed by developing efficient microorganisms capable of utilizing xylose, a major waste product of the paper industry [13]. P4HP has been studied in tissue engineering applications such as tri-leaflet heart valves, peripheral vascular applications, biodegradable suture material, and hernia repair [6, 14-16]. However, hemocompatibility testing of P4HB in past years has focused on platelet adhesion, rather than encompassing more systematic studies [17, 18]. The purpose of the present study was to investigate the hemocompatibility of P4HB more thoroughly to assess its safety and feasibility in tissue engineering, especially in cardiovascular system applications.

Materials and methods

Preparation of whole blood and platelet-rich plasma

Fresh whole blood was obtained from the median cubital vein of healthy volunteer donors who were drug-free for 7 days prior to the experiment, in accordance with approval from the ethics committee at Sun-Yat-sen University. Blood was collected into acid citrate dextrose (ACD, Nigale Biomedical Inc.; blood: ACD in a ratio of 9:1) anticoagulant and stored at 4°C for a maximum of 6 hours prior to use.

To obtain platelet-rich plasma (PRP), 50 mL of whole blood were mixed with 5 mL of 3.8% sodium citrate (Sigma) and centrifuged at 100 × g for 15 minutes. PRP was stored at 4°C for a maximum of 6 hours prior to use.

Preparation of the scaffold materials

P4HB (molecular weight: 1000 kDa, melting point: 60°C) was biosynthesized using the recombinant bacterial strain Bacillus subtilis 1A304. The P4HB material was dried in an oven at 50°C for 2 hours, after which 0.6 g P4HB was placed in a beaker and 8 mL of pure chloroform was added. The mixtures were molded into 5 cm × 5 cm thin films after satisfactory dissolution. Medical polyvinyl chloride (PVC), chosen as a suitable experimental control material for this study due to its good blood compatibility and wide usage in medical devices such as cardiac pulmonary bypass pipeline [19, 20], was obtained from medical blood storage bags cut into 5 cm × 5 cm sections. P4HB thin films and PVC membranes were cleaned and soaked in physiological saline (also called

normal saline, NS) for 12 hours, and then sterilized using ethylene-oxide gas after airing.

Hemolysis testing

Hemolysis was evaluated by determining the relative amounts of hemoglobin released into solution phase from erythrocytes in whole blood exposed to the test materials. P4HB film was cut into 5 mm × 10 mm sections. Sections were washed 3 times with NS. Eight milliliters of anticoagulated blood diluted with 10 mL NS provided the hemoglobin source. The test group comprised 5 siliconized test tubes containing P4HB in 10 mL NS. Five negative control group tubes contained 10 mL NS only. Five tubes containing 10 mL distilled water to induce maximal lysis of erythrocytes formed the positive control group. All test tubes were preheated in a 37°C constant-temperature water bath for 30 minutes, after which 0.2 mL diluted anticoagulated fresh whole blood were added, and the tubes replaced in the water bath for a further 60 minutes. Following the second incubation period, the test tubes were centrifuged at 100 × g for 5 minutes. The supernatant containing the solubilized hemoglobin was removed and its absorbance determined at a wavelength of 540 nm (O.D.). The average O.D. value was used to calculate the degree of hemolysis (%) according to the following formula: $(T_{sample}\text{-}T_{neg})/(T_{pos}\text{-}T_{neg}) \times 100\%$, where T_{sample} was the average absorbance of the test sample group, T_{neg} was the absorbance of negative control group, and T_{pos} was the absorbance of the positive control group [21].

Dynamic blood coagulation testing

Dynamic blood coagulation tests measured release of hemoglobin from residual erythrocytes that remained free from entrapment during clot formation. Assays were performed by recalcification of the anticoagulated whole blood, as described previously [22] with several modifications. Briefly, P4HB and PVC thin films were placed at the bottoms of individual 100 mL beakers and then prewarmed in a water bath at 37°C for 5 minutes. Subsequently, anticoagulated whole blood (0.25 mL) was dripped onto the surface of the films and incubated at 37°C for a further 5 minutes, after which $CaCl_2$ solution (0.02 mL of 0.2 mol/L) was dripped into the blood to initiate the coagulation cascade (time 0). The beakers were shaken for 1 minute to mix the $CaCl_2$ uniformly with the blood. Heating of the covered beakers was continued at 37°C for a predetermined time (10, 20, 30, 40, or 50 minutes). At the designated termination point, the beakers were removed from the waterbath and shaken for 10 minutes following addition of 50 mL distilled water to lyse the free erythrocytes. The absorbance of the supernatants at 540 nm was determined. Since the signal was derived from that proportion of erythrocytes remaining free of clot entrapment, absorbance was inversely proportional to the size of the clots. The absorbance-time curve was constructed using the average values from 5 replicate experiments.

Influence of scaffold materials on blood components

Adsorption of erythrocytes, leukocytes, and platelets from whole blood onto PVC and P4HB: The P4HB and PVC films were cut into rectangles (0.5 cm × 1.5 cm) and placed separately into 16 EDTA anticoagulation tubes (BD Vacutainer®, K2 EDTA 3.6 mg) for testing. Non-anticoagulated whole blood (3 mL) drawn immediately prior to the experiment was injected into each tube and maintained at room temperature for 30 minutes. The quantities of erythrocytes, leukocytes, and platelets remaining in the solution phase were determined in 8 of the tubes using the Automatic Biochemistry Analyzer (Sysmex SF-3000; Kobe, Japan) according to manufacturer's instructions. The quantity of each cell type adsorbed to the scaffold material was determined by subtraction of the appropriate solution phase values from the values found in control samples containing no scaffolding materials.

Morphology of adsorbed platelets: Surface-associated platelets may be passively adsorbed in a native state or adhered in an activated form, ready to participate in blood coagulation reactions as well as normal or pathological wound-healing processes. These states can be distinguished by the morphological appearance of the surface-associated platelets. Observation under the scanning electron microscope (SEM) was used to examine morphology. P4HB thin films were cut into quarters (1 cm × 1 cm) and immersed in fresh phosphate-buffered saline (PBS) solution for 1 hour, then placed in 24-well plates. One milliliter of PRP was added to each well. After an incubation period of 1

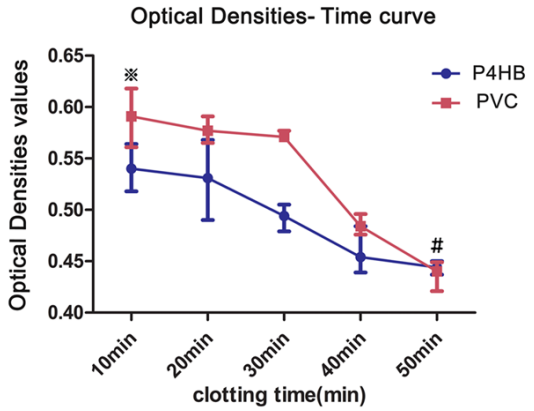

Figure 1. Dynamic clotting reactions suggest similar long-term procoagulant propensities for PVC and P4HB. Absorbance versus time was determined for hemoglobin release during dynamic clotting reactions in recalcified whole blood. Coagulating whole blood was allowed to entrap a proportion of the erythrocytes present during the indicated incubation times. Erythrocytes remaining in the solution phase were lysed and the absorbance of the released hemoglobin at 540 nm was determined. Data represent mean ± SD. Blue symbols, coagulation in the presence of P4HB. Red symbols, coagulation in the presence of PVC. P4HB, poly-4-hydroxybutyrate; PVC, polyvinyl chloride. *$P < 0.05$; #$P > 0.05$.

hour at 37°C, the P4HB films were removed, rinsed with PBS 3 times, and fixed with 2.5% glutaraldehyde for 30 minutes. All films were treated sequentially by freezing, drying, and gilding. Finally, the treated films examined by SEM to observe the adherent platelet morphology on the material surface.

Blood coagulation functional testing: The activity of the procoagulant protein, Factor XII (FXII), the activated partial thromboplastin time (APTT), and the content of fibrin degradation products (FDP) in whole blood samples exposed to scaffolding materials were determined using the Automated Coagulation Analyzer (TOA Medical Electronics Co., Kobe, Japan) according to the manufacturer's instructions. The ranges of the normal reference values are as follows: FXII, 71.7-133.1%; APTT, 25-35 seconds; FDP, 0-5 μg/mL.

Wright staining: Buffered Wright staining is useful to differentiate blood cell types in a sample. To identify blood cells that adsorbed to P4HB, microscope slides coated with thin films of this material were treated with buffered Wright stain. P4HB was dissolved in chloroform and poured onto microscope slides to form a hyaline thin film and dried in biological safety cabinets. The thin film was immersed in anticoagulated fresh whole blood (3.0 mL) for 30 minutes, then placed into PBS to wash off non-adherent hemocytes, fixed in methanol for 3 to 5 minutes, stained with Wright solution for 2 minutes, incubated with a Wright stain in phosphate buffer for 5 minutes, washed with distilled water, air-dried, and mounted with neutral gum prior to microscopic examination.

Quantities of plasma proteins adsorbed: Sections of P4HB and PVC films (0.5 cm × 1.5 cm) were placed separately into 6 EDTA anticoagulation tubes as the experimental group. Tubes lacking scaffolding materials comprised the control group. Three milliliters of anticoagulated fresh whole blood were injected into each tube and maintained at room temperature for 30 minutes. Then, the total plasma protein, albumin, and globulin content of the fluid fraction of each were measured using an Automatic Biochemistry Analyzer (Hitachi 7170A, Japan) according the manufacturer's directions. The blank control group contained no added film materials. The quantity of plasma protein adsorbed was calculated as equal to the difference between the plasma protein content in the blank control and experimental groups.

Statistical analysis: Data are presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to analyze significant differences. A Welch test was performed to verify that data were not subject to platykurtosis. A *P*-value < 0.05 was considered to be statistically significant. Calculations were performed using SPSSV 16.0 Stat. software (SPSS Inc., Chicago, IL, USA).

Results

Acceptable hemolysis in the presence of P4HB

The degree of hemolysis is a sensitive indicator of the extent of damage to erythrocytes. Absorbances in the hemolysis test for the samples exposed to P4HB, the positive control, and the negative control were 0.25 ± 0.01, 0.86 ± 0.02, and 0.23 ± 0.01, respectively. The degree of hemolysis in the presence of P4HB was therefore 1.9 ± 0.2%. Generally, a usable biomaterial should yield less than 2% hemolysis. A higher degree of hemolysis indicates poor hemocompatibility of the biomaterial. All mate-

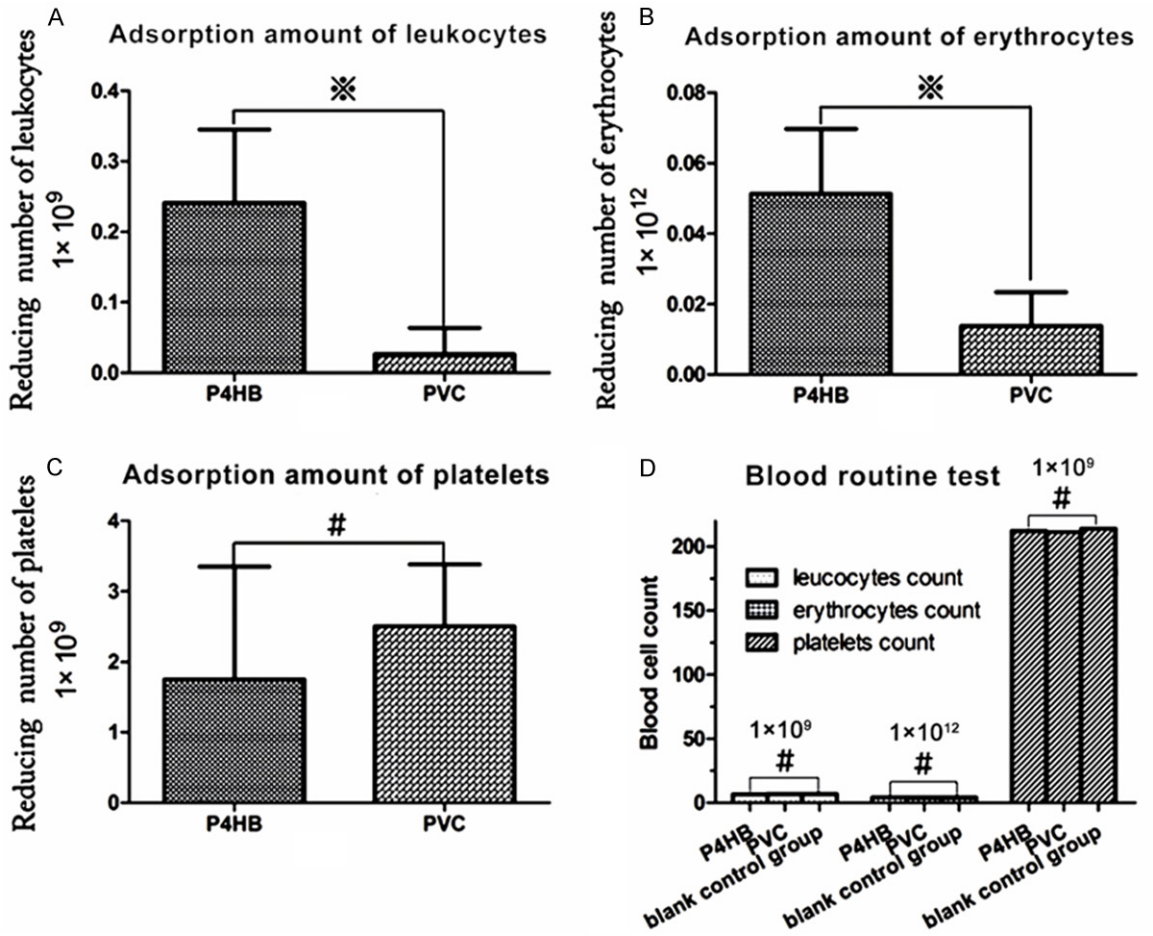

Figure 2. Differential adsorption of hemocytes onto PVC and P4HB. Anticoagulated whole blood was incubated in the presence of segments of the indicated scaffolding materials. The number of each cell-type adsorbed was determined by subtraction of the number remaining in the solution phase from that in the solution phase of control incubation without scaffolding material. Y-axes indicate the difference. A: Leukocytes; B: Erythrocytes; C: Platelets; D: Results of routine blood counts performed on the Automatic Biochemistry Analyzer. Data represent mean ± SD. P4HB, poly-4-hydroxybutyrate; PVC, polyvinyl chloride. *$P < 0.05$; #$P > 0.05$.

rials in current medical use, including PVC, meet this standard [23]. The data suggest that P4HB provides an acceptable level of hemolysis.

Moderate procoagulant stimulus by P4HB determined by dynamic blood clotting testing

The dynamic blood clotting time is an in vitro test that measures the degree of activation of intrinsic coagulation factors when surfaces interact with blood ("contact activation"). Activation is measured by the absorbance - time curve for release of hemoglobin from erythrocytes that avoid being entrapped during clot formation. A curve providing a gentle slope usually implies low procoagulant properties in the implanted material. **Figure 1** shows that the curves for P4HB and PVC are similar, with both presenting slow and smooth downward inclination; however, the slope of the P4HB curve is lower than that of PVC. The curves converge by the 50-minute time-point. Despite this, at earlier times clot sizes appear to be lower in the PVC samples, since the magnitude of absorbance is inversely related to clot size. This suggests that the procoagulant propensities of these 2 surfaces may not differ in the long-term.

P4HB and PVC interact similarly with blood components

Differential adsorption of erythrocytes, leukocytes, and platelets from whole blood onto PVC and P4HB: Deleterious interactions of implant-

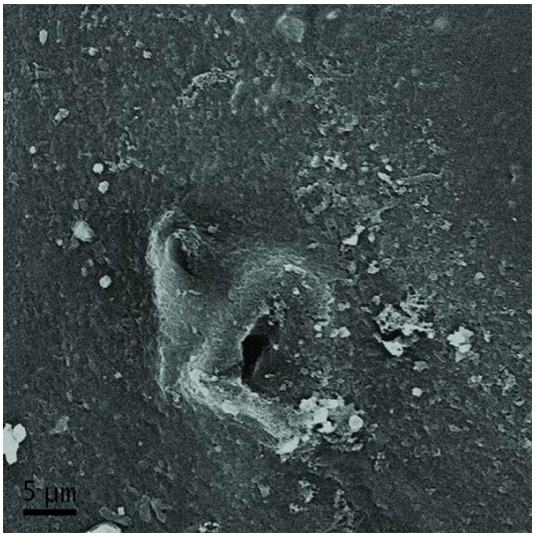

Figure 3. Scanning electron micrography showing few but unactivated platelets adhering to P4HB. Platelet-rich plasma was incubated with P4HB film as described in "Methods" and subjected to electron microscopy. The scale bar indicates a 5 μ measure. P4HB, poly-4-hydroxybutyrate.

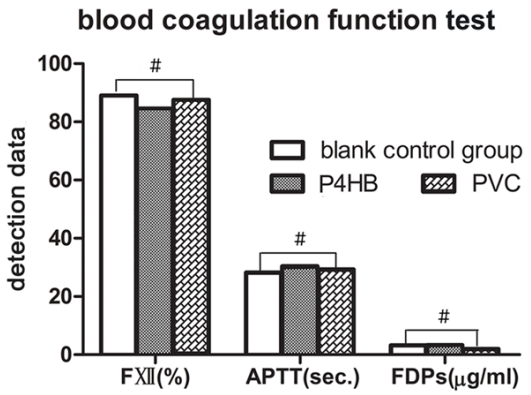

Figure 4. Lack of blood coagulation activation in the presence of PVC and P4HB. Blood coagulation parameters of anticoagulated blood samples incubated in the presence of the indicated scaffolding materials were determined using the Automatic Coagulation Analyzer. Data represent mean ± SD. The Y axis is unitless; respective applicable units are defined beneath each respective bar grouping. P4HB, poly-4-hydroxybutyrate; PVC, polyvinyl chloride; FXII, Factor XII activity; APTT, activated partial thromboplastin time; FDP, fibrin degradation products. *$P < 0.05$; #$P > 0.05$.

ed devices with blood may begin with deposit of cellular components onto their surfaces. To guide future research into the pathways activated by these interactions, it is necessary to understand the adhesion of these cells. **Figure 2** illustrates the relative adsorbance of various hemocytes from anticoagulated whole blood onto PVC and P4HB. The quantity of erythrocytes and leukocytes adsorbed differed greatly between the 2 groups, with P4HB adsorbing more in both cases. In contrast, the number of platelets adsorbed was not significantly different between the 2 groups ($P > .05$) (**Figure 2**). However, routine blood tests on the PVC, P4HB, and blank control groups exhibited no statistically significant differences ($P > 0.05$).

Minimal platelet adhesion to P4HB: Adhesion and activation promote platelet participation in procoagulant reactions. The process may differ from simple passive adsorption. Activated platelets undergo changes including surface invagination, movement of negatively-charged phospholipids to the outer leaflet of the membrane, expression of adhesion protein receptors, secretion of the contents of α-granules and dense granules, and other transformations. Certain of these changes are associated with altered morphology that can be easily observed in the SEM. P4HB film was exposed to PRP and examined by SEM. The SEM image shows that few platelets adhered to the P4HB film surface (**Figure 3**). The adherent platelets observed did not exhibit the significant deformation or extension of pseudopodia that is normally associated with activated status.

Lack of procoagulant activity in the presence of P4HB: Coagulation parameters were determined to explore the activation status of whole blood placed in contact with PVC or P4HB films. As shown in **Figure 4**, the FXII, APTT, and FDP test values of the experimental group were all within the range of the normal reference values (see "Methods") and there were no statistically significant differences in the samples exposed to either scaffolding material compared with the blank control group ($P > 0.05$). This suggests that activation of the blood coagulation cascade is no more of a concern with P4HB than it is with PVC, a scaffolding material currently in common use.

PH4B-associated hemocytes comprise mainly erythrocytes and neutrophils: The type of cells present on the scaffolding material dictates the fate of the biomaterial over time. P4HB film was coated onto microscope slides and exposed to whole blood samples followed by buffered Wright staining as described in "Methods". Staining revealed hemocytes adhered in the following proportions: 37.4% erythrocytes, 29.6% neutrophils, 14.8% leukocytes, and 18.2% platelets. Thus, most hemocytes

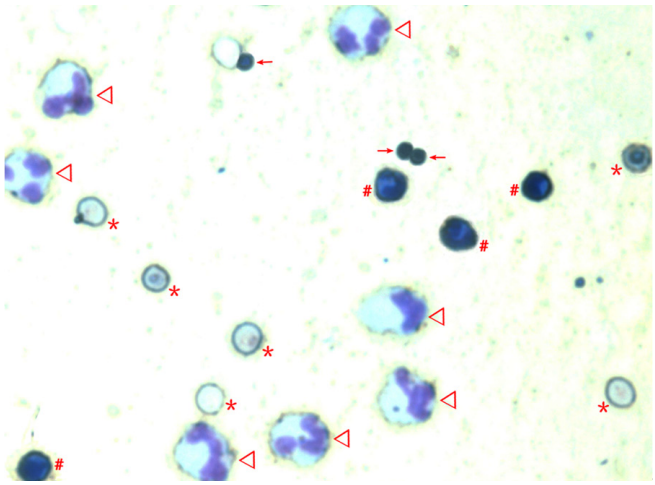

Figure 5. Predominance of erythrocytes and neutrophils adhering to P4HB. P4HB was coated onto microscope slides as a thin film and incubated with anticoagulated whole blood, then subjected to buffered Wright staining as described under "Methods". P4HB, poly-4-hydroxybutyrate. *Erythrocytes; →, platelets; #, lymphocytes; Δ, neutrophils. Magnification is x1000.

adsorbed onto the P4HB film surface were neutrophilic granulocytes (a type of leukocyte) and erythrocytes, while lymphocytes and platelets were seldom adsorbed (**Figure 5**). Neutrophils are able to release inflammatory cytokines and superoxide free radicals that may be either harmful to the host or beneficial to the process of resorption of the scaffolding material over time.

Protein coating of P4HB exposed to blood appears benign: Protein adsorption is known to mediate cell interactions with scaffolding materials, and thus to promote the eventual replacement of the synthetic material with biological constituents. As described under "Methods", PVC and P4HB films were allowed to interact with anticoagulated whole blood prior to protein analysis of the non-adsorbed fraction. The relative levels of each protein in the 3 incubations are shown in Panel 4 for reference. As shown in **Figure 6**, the quantities of total plasma protein and albumin adsorbed in the P4HB group were higher than those in the PVC group ($P < 0.05$). The amount of globulin bound was similar between the P4HB and PVC groups ($P > 0.05$). Therefore, P4HB may be predicted to be at least as good an environment for colonizing cells as PVC. The amounts of each type of protein remaining in the solution-phase fraction remained the same among scaffold-incubated and control samples (**Figure 6D**), suggesting that local depletion of plasma factors would not be an issue.

Discussion

The biosynthetic polymer, P4HB, has been studied as a new implantable medical polymer material in tissue engineering in recent years. Our previous work [24] demonstrated that the porosity of P4HB films is an important aspect influencing their biodegradation rates, but the biocompatibility of these films has not been systematically studied. Previous work has examined the propensity of polyhydoxyalkanoates to induce thrombosis in vivo and to activate platelets in clotting time tests and by morphological changes [18, 25]. Here, we performed a series of in vitro experiments to evaluate several aspects of the hemocompatibility of P4HB film. These experiments evaluated the ability of P4HB to evoke hemolysis of erythrocytes, shorten the clotting time of blood, and adsorb circulating blood cells and plasma proteins, and thus extended the analysis of blood interactions beyond the aspects previously reported.

Biomaterials that are toxic to erythrocytes can destroy the erythrocyte membranes and release hemoglobin, which in turn can induce acute renal failure [26]. The degree of erythrocyte hemolysis is 1 of the most important factors in evaluation of the biocompatibility of biomaterials [21]. The degree of hemolysis in the presence of P4HB was 1.9% (see above), which meets the requirement for medical biomaterials (< 2%) [23], suggesting that P4HB does not cause undue destruction of red blood cells.

Dynamic blood clotting testing is a common method used in the biomaterials field to detect the activation of intrinsic blood coagulation factors for the purpose of evaluation of the overall effect of bioengineering materials on coagulation times. The absorbance - time curves of the dynamic blood clotting time for PVC and P4HB were similar. Both tilted down slowly, and maximal clotting required a long period of time (> 50 minutes), which indicated that the materials have suitably low procoagulant properties (**Figure 1**) [27].

Low procoagulant activity may be verified and shown more specifically by tests of intrinsic

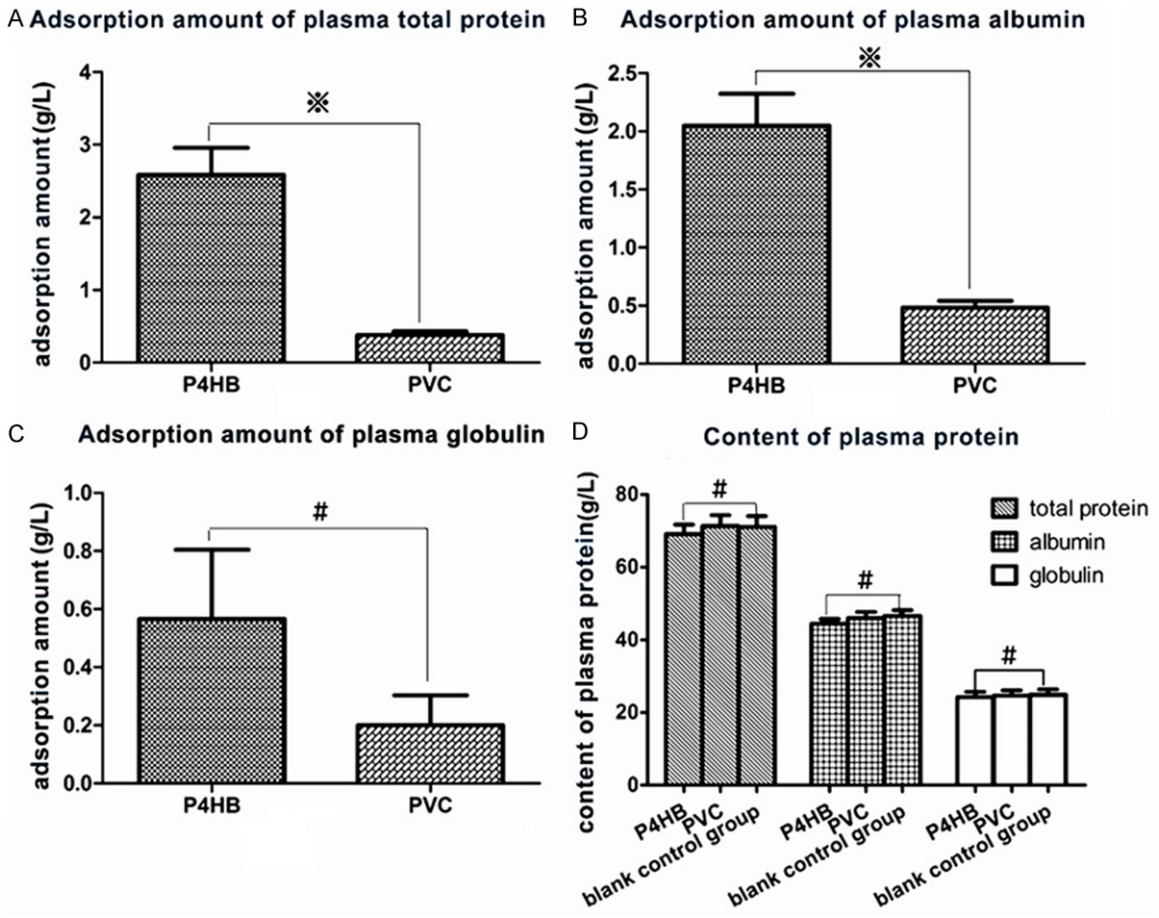

Figure 6. Adsorption of plasma proteins onto PVC and P4HB. Anticoagulated whole blood was incubated in the presence of the indicated scaffolding material. The amount of each protein adsorbed was calculated by subtraction of the amount remaining in the solution phase from that in the solution phase of the control sample as described in "Methods". A: Adsorbed total protein; B: Adsorbed plasma albumin; C: Adsorbed plasma globulin; D: Raw protein levels in each preparation. Data represent mean ± SD. P4HB, poly-4-hydroxybutyrate; PVC, polyvinyl chloride. *P < 0.05; #P > 0.05.

pathway activation and subsequent clot dissolution. The intrinsic coagulation pathway that is commonly initiated by implants begins with the activation of coagulation FXII. Zymogen FXII can be activated and transformed into FXIIa when in contact with exogenous surfaces ("contact activation"). This activation leads to a cascade of sequential proteolytic coagulation factor activation reactions in plasma [28]. The APTT provides a comprehensive index for the overall activation of intrinsic pathway coagulation factors. A lessened propensity for procoagulant processes causes the prolongation of APTT [29]. After the activation of the coagulation pathway leading to clot formation, mechanisms are initiated to dissolve the resulting thrombus. The zymogen, plasminogen, is converted into plasmin, which breaks down fibrin into fibrin degradation products (FDPs) [30]. Levels of FDP are a sensitive indicator of ongoing coagulant processes and increase concurrently with ongoing and coagulation and fibrinolysis. In this study (**Figure 4**), the activity of FXII, the APTT values, and the content of FDPs in whole blood allowed to contact P4HB underwent almost no changes compared with samples in the blank control group (P > .05). These results indicate that P4HB has minimal influence on clotting factors and the fibrinolytic system.

Adsorption of blood cells to bioengineering materials exposed to the circulation dictates the pathways of tissue regeneration and ultimate replacement of the synthetic material with cellular materials. Many steps in this path-

way remain to be elucidated, but new materials must be investigated for their properties in this regard. Numerical reduction of erythrocytes and leukocytes remaining in the solution phase relative to a control was used to estimate the degree of adsorption onto scaffold materials during incubation with whole blood samples. Adsorption was greater in the P4HB group than in the PVC group ($P < .05$), implying greater adherence of these cells to the P4HB film surface. Wright staining showed that hemocytes adsorbed on the P4HB film surface consisted mainly of erythrocytes and neutrophilic granulocytes. The quantity of adsorbed platelets exhibited no significant difference between the 2 groups. Platelets in contact with materials of poor hemocompatibility become seriously distorted (activation-induced "shape change") and display extended pseudopodia, and may even form thick reticulate structures with fibrinogen that deposit on the surfaces of such incompatible foreign bodies [31]. Few platelets adhered to the P4HB film surface (**Figure 5**), and under SEM imaging (**Figure 3**), the adherent platelets from PRP exhibited no significant deformation or extension of pseudopodia indicative of activation. Consequently, P4HB appears to demonstrate good compatibility with platelets, and therefore appears unlikely to be prothrombotic. Deposition of erythrocytes and neutrophilic granulocytes on the P4HB film surface may be promoted by characteristics of its surface chemistry or by the formation of a 3-D structure. However, it appears that the P4HB merely induced the adhesion of erythrocytes without serious damage to the cell membrane, to judge by the low hemolysis rate (1.9%). Leukocytes, especially neutrophilic granulocytes, have strong chemotactic stimulus properties, encouraging their emigration to and retention by the surfaces of exogenous substances [32]. An activated neutrophilic granulocyte can release many enzymes and inflammatory factors that induce an inflammatory response [33]. This inflammatory response is sometimes severe, causing damage to organs or even systemic reactions such as fever, arrhythmia, acute lung injury, chronic inflammation, etc. The significant neutrophil adsorption onto P4HB suggests that P4HB might promote an inflammatory response. This may be deleterious, but could also participate in cellular enclosure of the synthetic surface. However, examination of neutrophil activation and release of inflammatory cytokines is beyond the scope of the current study.

Hemocompatibility is determined by the reactions that occur at the interface between blood and a biomaterial. The deposition of different proteins on the material surface has been shown to occur in a sequential and competitive manner and to trigger different responses [34]. It was previously thought that the adsorption of albumin on the surface of an implant enhances its anti-thrombogenic properties and suppresses platelet adhesion [35, 36]. However, platelet adhesion has recently been observed to occur even on albumin-coated surfaces, which may be an effect of the adsorption-induced loss of a percentage of the α-helix in albumin [37].

The adsorption of γ-globulin on the surface of a material promotes the adhesion of platelets because these plasma proteins specifically bind to platelet membrane glycoproteins [35]. We observed (**Figure 6**) that the quantity of plasma globulin adsorbed to P4HB was similar to that adsorbed to PVC ($P > 0.05$). By contrast, the quantity of plasma albumin adsorbed to P4HB was much higher than that adsorbed to PVC ($P < 0.05$). Few platelets adhered to the P4HB film. As discussed above, P4HB had minimal influence on the procoagulant and fibrinolytic systems, consistent with the conclusion that no critical conformational changes occurred in the adsorbed plasma proteins.

P4HB has been previously reported to show satisfactory low procoagulant activity when impregnated into decellularized aortic matrix and implanted into rabbits [38]. In contrast, other polyhydoxyalkanoates, including poly 3-hydroxybutyrate and 3-hydroxyvalerate, were relatively inert with respect to platelets but were reported to activate the coagulation and cascade [18]. It has also been noted that the copolymer poly (3-hydroxybutyrate-co-4-hydroxybutyrate) both shortens clotting time and promotes platelet adhesion [39]. The presently reported data concur with Stamm and coworkers [38] in showing a similar lack of platelet activation with P4HB, but diverge from both of these studies in showing no evidence of coagulation cascade activation. These discrepancies may result from differences in the properties of P4HB that may redound on its utility for specific medical applications.

Limitations of this study include the constraint of its scope to gross examination of cellular adhesion to the surface (leaving the study of molecular mechanisms to future work), its in vitro nature, and the static incubation conditions of the blood with the surfaces, absent the hemodynamic fluctuations present *in vivo*.

In conclusion, P4HB has minimal toxicity for erythrocytes (judged by low level of hemoglobin release) and a minimal influence on clotting factors and the fibrinolytic system (judged by lack of perturbation of measured coagulation system parameters, **Figure 3**) or other plasma proteins (judged by the lack of effect on concentration of globulins, albumin, or total plasma proteins, **Figure 6**). In addition, the P4HB membrane had an affinity for erythrocytes and leukocytes, especially for neutrophils, but not for platelets. Directions for future research include analysis of participation of P4HB in inflammatory processes and the contributions of the adherent leukocytes to specific neotissue formation processes and biodegradation of the scaffolding. Understanding of these interactions will guide future design improvements in this and other scaffolding materials.

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Disclosure of conflict of interest

None.

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