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ORIGINAL PAPER

Effects of various monomers and micro-structure of polyhydroxyalkanoates on the behavior of endothelial progenitor cells and endothelial cells for vascular tissue engineering

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Abstract Cardiovascular diseases are a leading cause of mortality in the world today. Vascular tissue engineering is an important and attractive research issue for the repair and regeneration of blood vessels. Two bio-based polymers, poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), which both belong to the polyhydroxyalkanoate (PHA) family, were used in this study. The aim of this study is to assess the potential application of PHB and PHBV to serve as a scaffold that is seeded with human umbilical vein endothelial cells (HUVECs) or endothelial progenitor cells (EPCs) for vascular tissue engineering. PHA films with various surface characteristics were prepared by solution-casting (surface roughness) and electrospinning (mesh-like structure). First, the mechanical and physical properties of various types of PHA films were analyzed. Then, the PHAs films were examined for cytotoxicity, biocompatibility and proliferation ability using cell lines (3 T3 and L929) and primary cells (HUVECs and EPCs). The cell morphology cultured on the PHA films was observed by fluorescence microscope and scanning electron microscopy. In addition, cultured EPCs on various types of PHA films were analyzed for whether the cells maintained the abilities of Ac-LDL uptake and UEA-1 lectin binding and exhibited specific gene expressions, including VEGFR-2, vWF, CD31, CD34 and CD133. Importantly, the cell retention rate and anti-coagulation ability of HUVECs or EPCs cultured on the various types of PHA films were also evaluated at the indicated time points. Our results showed that PHA films that were prepared using electrospinning methods (Ele-PHB and Ele-PHBV) had good mechanical and physical properties. HUVECs and EPCs can attach and grow on Ele-PHB and Ele-PHBV films without showing cytotoxicity. After a one-week culture, expanded HUVECs or EPCs maintained the correct cell morphologies and exhibited correct cell functions, such as high cell attachment rate and anti-coagulation ability. Taken together, Ele-PHB and Ele-PHBV films were ideal bio-based polymers to combine with HUVECs or EPCs for vascular tissue engineering.

Keywords Polyhydroxyalkanoates · Casting · Electrospinning · Endothelial progenitor cells · Vascular tissue engineering

This article is part of the Topical Collection on *Bio-Based Polymers*

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Abbreviations

A540	The absorbance at 540 nm
Ac-LDL	Acetylated low-density lipoprotein
Cast-PHB	Solvent-cast PHB
Cast-PHBV	Solvent-cast PHBV
DAPI	4′,6-Diamidine-2′-phenylindole dihydrochloride
DMEM	Dulbecco's modified Eagle's medium
DMF	N,N-dimethyl formamide

EC	Endothelial cells
ECGS	Endothelial cell growth supplement
ECM	Extracellular matrix
Ele-PHB	Electrospun PHB
ElePHBV	Electrospun PHBV
EPC	Endothelial progenitor cell
F-actin	Filamentous actin
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPC	Gel permeation chromatography
HUVEC	Human umbilical vein endothelial cell
HV	Hydroxyvalerate
LSS	Laminar shear stress
MNC	Mononuclear cell
Mw	The weight-averaged molecular weight
PCR	Polymerase chain reaction
PDI	The polydispersity indice
PHA	Polyhydroxyalkanoate
PHB	Poly(3-hydroxybutyrate)
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
SEM	Scanning electron microscopy
TCPS	Tissue culture polystyrene
UCB	umbilical cord blood
UEA-1 Lectin	FITC-conjugated Lectin from *Ulex europaeus*
vWF	von Willebrand factor

Introduction

One of the top ten lethal diseases is cardiovascular disease. The coronary arteries of the elderly often suffer from chronic and acute vascular obstruction due to atherosclerosis [1]. The coronary artery is the one of the most important blood vessels that supplies oxygen and blood to the heart. If the heart cannot get enough nutrients and oxygen, the result is myocardial damage and impaired myocardial function; severe cases will be life threatening. In the current medical setting, the most common coronary artery occlusion treatments are stents and bypass surgery. Bypass surgery is currently the only treatment option for severe cases, but patients who undergo the treatment may face recurrence of the disease due to re-blocking of the vessel [2–5]. Stent implantation can change the mechanical properties near the area of implantation [6]. The veins implanted during bypass surgery are subjected to arterial blood pressure, which changes the mechanical environment and can result in damage and affect the function of endothelial cells and smooth muscle cells, especially their proliferation and migration [7, 8].

To solve these problems, vascular tissue engineering has been developed in recent years [9]. The technology contains three factors: cells, stents, and stimulation. The vascular cells can be categorized into endothelial cells, smooth muscle cells and fibroblasts [10, 11]. As for stents, they can be classified as natural scaffolds and polymer scaffolds. Finally, there are physical and chemical types of stimulation [12, 13].

In this study, the major cells of vascular tissue, endothelial cells (ECs) and endothelial progenitor cells, were selected to be cultured on a bio-based polymer scaffold. EPCs can be harvested from bone marrow [14, 15], peripheral blood [16, 17] and cord blood [18–20] and are progenitor cells with the ability to differentiate into ECs [21, 22]. If EPCs are cultured in endothelial induction medium, they have the ability to differentiate and mature into functional ECs, and they can expand more than several billion-fold with a consistent population doubling time to generate large amounts of ECs for tissue engineering applications [23–28].

The ideal scaffold should be biocompatible and biodegradable and should have the appropriate degradation and adsorption rates for tissue replacement [10, 11, 29]. Scaffolds can be prepared by many methods, such as the traditional solvent casting method and the emerging static electricity spinning technology [30]. Electrospinning can produce a scaffold into which cells can penetrate more easily through porous holes and that has better connectivity. The connectivity is conducive to cell attachment through the use of fiber shape to guide the growth direction of cells, thus producing a similar extracellular matrix for good artificial blood vessels that contain good biocompatibility, good biodegradability and a surface for improved cell attachment [31–33]. Polyhydroxyalkanoates (PHAs) are a superfamily of intracellular polyesters produced by microorganisms when uptake is in excess of the carbon source of nutrition. Over 150 kinds of PHAs with various co-monomers have been identified [34–42]. According to the literature, PHAs are biocompatible, biodegradable, nontoxic and exhibit good thermal and mechanical properties [43–48].

In this study, two members of the PHAs family, poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), were selected as bio-based polymers to fabricate various types of scaffolds based on the above criteria. The aim of this study is to assess the potential application of PHB and PHBV scaffolds seeded with ECs/EPCs for vascular tissue engineering. PHB and PHBV films were prepared using solution casting and electrospinning. The microstructure, mechanical and physical properties, cytotoxicity, biocompatibility, and cell adhesion ability of various PHA films were examined. After EPCs or ECs were cultured on

the various PHA films, the cell morphology, endothelial lineage-related gene expression, cell retention rate and anti-coagulation ability were evaluated at indicated time points. Our results showed that various PHA films prepared using solution casting and electrospinning had good mechanical and physical properties. ECs and EPCs can attach and grow on various PHA films without showing cytotoxicity. After a one-week culture, expanded ECs or EPCs maintained the correct cell morphologies and exhibited correct cell function. Taken together, PHB and PHBV films were ideal bio-based polymers to combine with ECs/EPCs for vascular tissue engineering.

Materials and methods

Preparation of PHA films

Two members of PHAs, PHB and PHBV (5% of the molar content of hydroxyvalerate (HV) in the co-polymers), were purchased from Sigma (St. Louis, MO) and were selected to fabricate the various types of films as scaffolds in this study. According to the gel permeation chromatography (GPC) assay, the weight-averaged molecular weights (Mw) of PHB and PHBV were 6.85×10^5 and 4.96×10^5, respectively, and the polydispersity indices (PDIs) of PHB and PHBV were 2.28 and 1.92, respectively.

Cast films were prepared by dissolving 3 wt% of PHB or PHBV in chloroform, which was then poured onto a glass plate. A film-casting knife (Braive) was pulled over the solution with controlled clearance to adjust the thickness. After the solvent evaporated in the air at room temperature for 24 h, a cast PHB or PHBV membrane was obtained.

Fibrous PHA films were produced using electrospinning. The 5 wt% PHB or PHBV was dissolved in an organic solvent mixture of chloroform and N,N-dimethyl formamide (DMF, Tedia). The polymer solution was loaded in a syringe capped with a 21-gauge metal needle. An electric field was created by a power supply at 12 kV between the needle and the rectangular stainless-steel receiver at a distance of 25 cm. The polymer solution was drawn from the needle under an accurate controlled syringe pump and then splayed onto the receiver by the combined forces of gravity and electrostatic charge. [49].

The apparent thickness of those films was determined by a digital thickness gauge (Mitutoyo, IDF-112) in an average of 10 measurements at different points of the films. The apparent thickness of the solvent-cast and electrospun films was approximately 60–70 μm. A variety of PHA membranes, including solvent-cast PHB (Cast-PHB), solvent-cast PHBV (Cast-PHBV), electrospun PHB (Ele-PHB) and electrospun PHBV (Ele-PHBV) films, were washed with deionized water three times to remove the endotoxin and then immersed in an aqueous solution of fibronectin (BD Biosciences, San Jose, CA) at room temperature for 1 h for the cell culture and further application.

Tensile test

The tensile properties of the various PHA films were measured in an Instron 3866 (Norwood, MA). Bone-shaped cut films (width: 2.2 mm, length: 20 mm and thickness ~ 70 μm) were assayed for each sample studied with a preload of 0.3 N at 5 mm min^{-1} and at room temperature. The results represent the average of three measurements.

Contact angle measurement

Contact angles of various PHA films were measured using a contact angle meter (KSV Instruments Ltd., Finland) using the sessile drop method at room temperature and an air atmosphere. Twenty microliters of deionized water was gently dropped onto the surface of various PHA films. Pictures of the water droplets were captured by a video camera (SINDATEK 100SB), and the contact angle between the droplet and the film surface was determined by measurements at five different parts of the films.

Cell preparation and cell culture on the PHA films

A variety of PHA membranes were placed in 24-well tissue-culture plates (BD Biosciences). The membranes were cut into 12.5-mm-diameter discs and were tightly wedged into the culture wells. To avoid the membrane floating in the medium, Teflon O-rings were used to fix the membranes to the bottom of the wells.

In this study, 3 T3 (BCRC 60071) and L929 (also named NCTC clone 929, BCRC 60091) cell lines were purchased from Bioresource Collection and Research Center, Taiwan and were selected as the standard cell lines to check the cytotoxicity and biocompatibility of the various PHA films. The culture medium for the 3 T3 cell line was Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 4 mM L-glutamine and 4.5 g/L glucose. The culture medium for the L929 cell line was alpha-MEM containing 10% FBS.

In addition, HUVECs and EPCs were selected as the primary endothelial cells and stem cells to evaluate the potential application of various PHA films for vascular tissue engineering. HUVECs were purchased from BCRC, Taiwan, and their culture medium was Medium 199 containing 10% FBS, 25 U/ml heparin and 30 μg/ml endothelial cell growth supplement (ECGS). EPCs were derived from human umbilical cord blood (UCB) with consent from the mother and after approval from the Institutional Review Board (Taoyuan General Hospital, Ministry of Health and

Welfare, Taiwan). Briefly, the buffy coat was obtained from UCB by centrifugation (700 x g for 20 min). The buffy coat cells were then layered onto Ficoll-Paque solution (1.077 g/ml; Amersham; Uppsala, Sweden) and centrifuged to deplete the residues of red blood cells, platelets, and plasma (700 x g for 40 min). Mononuclear cells (MNCs) at the interface were collected, washed twice with D-PBS (Sigma), seeded at a concentration of 10^6 cells/cm^2 in EGM-2 medium (Lonza Inc. Allendale, NJ) on the fibronectin (2 μg/cm^2)-coated T25 flask, and cultured at 37 °C in a humidified atmosphere with 5% CO_2. The non-adherent cells were removed using a medium change 3 days after seeding, and the medium was changed every 3 days. When well-developed colonies of endothelial-like cells appeared, cells were washed with PBS, harvested with 0.05% trypsin-EDTA (Sigma) and passed into new T75 flasks. EPCs (expressing CD34) and HUVECs (not expressing CD34) were identified by surface markers, including human CD31, CD34, CD105 (VEGFR2), CD309, and von Willebrand factor (vWF) using a BD Accuri™ C6 Flow Cytometer (BD Biosciences) [23, 50–52]. A replicate sample was stained with mouse IgG1 antibody as an isotype control to ensure specificity (data not shown).

The initial seeding cell density of 3 T3, L929, HUVECs or EPCs was 1×10^4 cells per film in a 24-well plate. With gentle shaking, the cells were spread over the surface of the PHA membranes. The cells were cultured in a humidified incubator in the presence of 5% CO_2 at 37 °C. The cell number and behavior were analyzed at the indicated time points.

Structural property and cell morphology assay: scanning electron microscopy assay

The microstructures (including the average diameter of the pores and fiber) of the electrospun PHB and electrospun PHBV films and the cell morphology of the 3 T3, L929, HUVECs and EPCs cultured on the electrospun PHA films were measured by the by the as-built software of scanning electron microscopy (SEM). After culturing on the various PHA films, the cells were fixed with 4% glutaraldehyde (Sigma) and dehydrated stepwise with mixtures of ethanol and water that were progressively richer in alcohol. Then, the cells on the PHA films were sputter coated with gold for 100 s and observed under an SEM operated at 10 kV.

Biocompatibility and cytotoxicity: WST-1 assay

The cell lines (3 T3 and L929) or two primary cell types (HUVECs and EPCs) were selected to be cultured on the surface of various PHA films. After culture, the biocompatibility and cytotoxicity of the PHA films were determined based on the cell numbers using a cell proliferation assay. At the indicated time points, the culture medium in the 24-well was removed, and 10 μl of PreMix WST-1 mixture solution (TAKARA Bio Inc., Japan) was added to react with cells for 4 h at 37 °C in a CO_2 incubator. Then, the absorbance of each sample was measured using a micro plate reader (Epoch, BioTeK, Winooski, VT, USA) at a wavelength of 450 nm.

Cell staining

After 1, 3 or 7 days of incubation, the culture medium was removed from each dish, and the cells on the PHA film were washed with D-PBS. Subsequently, the cells on the various PHA films were fixed with 4% paraformaldehyde (Sigma) for 10 min, washed with PBS, and then permeabilized with 1% Triton X-100 (Sigma) for 15 min. After treating, cells were rinsed with PBS and then incubated with 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI, Biolegend, San Diego, CA) and filamentous actin (F-actin, Thermo Fisher Scientific Inc., Waltham, MA) for 20 min to label cell nuclei and cytoskeleton of the cells, respectively. To exam the cell function, HUVECs and EPCs were further stained with Dil-labeled acetylated low-density lipoprotein (Ac-LDL, Invitrogen, Waltham, MA) and FITC-conjugated Lectin from *Ulex europaeus* (UEA-1 Lectin, Sigma) for 30 min at 37 °C. After the incubation, the cells were washed three times with PBS, and fluorescence images were obtained using a fluorescence microscope (Zeiss Axiovert A1, Oberkochen, Germany).

Real-time polymerase chain reaction (PCR) assay

Total RNA was isolated from EPCs and HUVECs using TRIzol reagent (Ambion, Waltham, MA). The RNA was reverse transcribed into a first-strand DNA using a PrimeScript™ RT reagent kit (TAKARA Bio Inc.) according to the manufacturer's instructions. For real-time PCR, primers of CD31, CD34, CD133, VEGFR-2, vWF and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, housekeeping gene for normalization) were used [53]. The primer sequences are listed in Table 1. Real-time PCR with SYBR Green Mix (Thermo Fisher Scientific) was carried out on a StepOne™ real-time PCR system (Thermo Fisher Scientific). The specificity of the primers was confirmed by the single peak in the melting curve. Each target mRNA level was evaluated from the real-time threshold cycle and compared with GAPDH as an internal control.

Cell retention evaluation

To mimic the blood vessels in vivo and test the attachment ability between cells and PHA films, 5×10^4 per film of HUVECs or EPCs were seeded on various types of PHA films and were cultured statically in the 5% CO_2 incubator overnight to ensure that the cells attached to the surface of

Table 1 Target gene primer sequences

Gene	Sequence (5′ to 3′)	
CD31	Forward primer	CCTGATGCCGTGGAAAGC
	Reverse primer	TCCAGGGATGTGCATCTGG
CD34	Forward primer	GAAATTGACTCAGGGCATCTGC
	Reverse primer	TCTTAAACTCCGCACAGCTGG
CD133	Forward primer	TGGATGCAGAACTTGACAACGT
	Reverse primer	ATACCTGCTACGACAGTCGTGG
VEGFR-2	Forward primer	ACCAGACGGACAGTGGTATGG
	Reverse primer	AGTGATATCCGGACTGGTAGCC
vWF	Forward primer	CAGGAAGACCACCTGCAACC
	Reverse primer	AAGCCGTAGGCAAACATCTCC
GAPDH	Forward primer	TGCACCACCAACTGCTTAGC
	Reverse primer	GGCATGGACTGTGGTCATGAG

the PHA films. Then, the PHA films with cells were rolled up and placed into glass tubes (6 mm in diameter). The surface of PHA films with cells was faced inward so that the cells could contact the fluid flow. Subsequently, the cells were exposed to continuous steady laminar shear stress (LSS) for 3 h at 20 dyne/cm^2, which is the peak shear stress in vivo. The fluid was the cell culture medium, and the fluid flow was generated by a peristaltic pump. Cell retention evaluation at the indicated time points was analyzed by counting cell numbers remaining rate on PHA films via WST-1 assay and staining cells with F-actin and DAPI via an inverted fluorescence microscope (Zeiss Axiovert A1) observation.

Coagulation test: kinetic clotting test

UCB was collected in a 15-ml tube for the coagulation test. Before the coagulation test, 1×10^4 cells per film of HUVECs or EPCs were seeded on the various types of PHA films in 24-well plates and then incubated at 37 °C for 3 days. After a 3-day culture, the culture medium was removed and the cells were washed by PBS twice. Then, 2 ml of the UCB was added into each well to contact with the PHA films containing cells. Non-cell-coated PHA films and Eppendorf tubes served as controls. To initiate the blood coagulation process, 0.25 M calcium chloride solution was added to the citrated blood samples. After reacting, the UCB sample was transferred into a 15-ml tube containing 5 ml of distilled water. Red blood cells were distended up by the hypotonic solution and released hemoglobin. The suspension red blood cells that had not been trapped in a thrombus were hemolyzed, whereas free hemoglobin was released in water. The concentration of the free hemoglobin released in the water was colorimetrically measured at 540 nm wavelength using a plate reader.

Statistical analysis

All the data for three repetitions in this experiment were evaluated by one-way ANOVA and compared using all pairwise multiple comparison procedures (Student-Newman-Keuls method) packaged in the SigmaPlot system (version 12.0). A p-value <0.05 was considered significant.

Results

Preparation and properties of PHA films by solvent-casting and electrospinning methods

In this study, two bio-based polymers, PHB and PHBV, were selected to fabricate the solvent-casting (Cast-PHB and Cast-PHBV) and electrospun (Ele-PHB and Ele-PHBV) films for testing the potential application of vascular tissue engineering. The casting films were manufactured by the solvent casting method, and electrospun nanofiber films were prepared through the electrospinning device. The surface properties, mechanical properties and water contact angles of the films, which are important factors for cell attachment and cell growth, were analyzed.

The surface properties and micro-structure of these films were observed by SEM (Fig. 1a). The surfaces of the Cast-PHB and Cast-PHBV films were rough and contained some cavities and bulges. The rough surface may result from the higher casting temperature. It is worth noting that these pores (approximately 1–2 μm in diameter) existed only near the surface and were not interconnected with each other. The Ele-PHB and Ele-PHBV films showed a non-woven mat structure. The average diameter of the Ele-PHB fibers (1.0–1.5 μm) is slightly larger than Ele-PHBV fibers (0.5–1.0 μm). In addition,

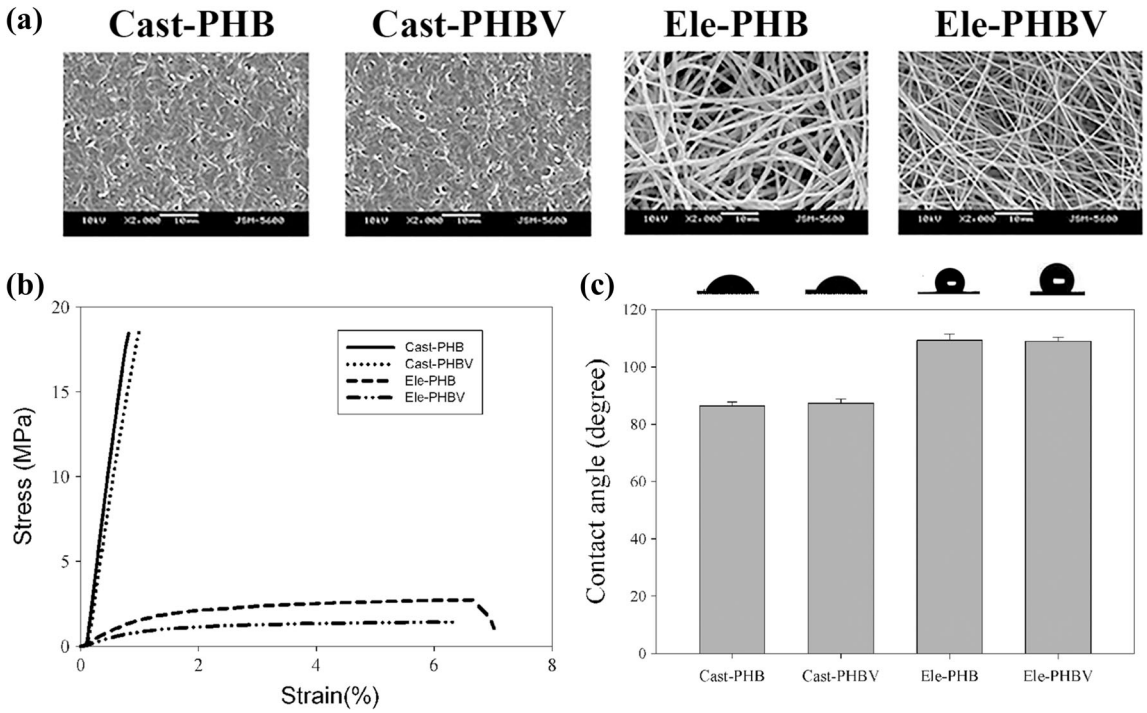

Fig. 1 Microstructure and physical properties of PHB and PHBV. **a** Microstructure of Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV under SEM observation. Scale bar is 10 μm. **b** Stress-strain relationship of Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV. **c** Contact angle measurement of Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV

the electrospun films had micropores that can facilitate cell growth.

The mechanical properties of these films were determined by a tensile test (Fig. 1b and Table 2). Electrospun films (Ele-PHB and Ele-PHBV) showed the higher ultimate strain and lower ultimate tensile than solvent-casting films (Cast-PHB and Cast-PHBV). That means that electrospun films are more suitable for vascular tissue engineering due to their elasticity and flexibility.

The water contact angle assay showed that the contact angles of PHA films depended on the different preparation methods. The contact angle of Cast-PHB and Cast-PHBV films were approximately 84°, while those of Ele-PHB and Ele-PHBV films were approximately 110° (more hydrophobic than the Cast films).

Cytotoxicity assay of PHA films by 3 T3 and L929 cell lines

The biocompatibility and cytotoxicity of Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV films were checked by the culture of 3 T3 and L929 cell lines (both are standard cell lines for cytotoxicity assays) on the surface of films and were evaluated by the WST-1 assay and DAPI staining (Fig. 2). The WST-1 assay and DAPI staining were used to evaluate the rate of cell viability and to measure the relative proliferation activity of the cells. The DAPI stained the cell nucleus blue and represented the cell numbers. Within 7 days of culture, WST-1 assay showed that both 3 T3 (Fig. 2a) and L929 (Fig. 2b) cell lines seeded on the Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV films can grow continuously. DAPI staining also showed an increase in the number of cells after 7 days of culture on the various types of PHA films (Fig. 2c and d). In addition, it is worth noting that cells cultured on the Ele-PHB and Ele-PHBV films exhibited a slightly higher growth rate than those on the Cast-PHB and Cast-PHBV films. The morphologies of 3 T3 and L929 cell lines cultured on the Ele-PHB and Ele-PHBV films were normal (Fig. 4). This meant that the Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV films are biocompatible and did not cause cytotoxicity.

Table 2 Mechanical properties of various types of PHA films

Mechanical property	Cast-PHB	Cast-PHBV	Ele-PHB	Ele-PHBV
Ultimate tensile (MPa)	18.44 ± 1.4	18.68 ± 0.96	1.63 ± 0.11	1.42 ± 0.09
Ultimate Strain (%)	0.81 ± 0.02	1.01 ± 0.08	7.01 ± 0.42	6.41 ± 0.31

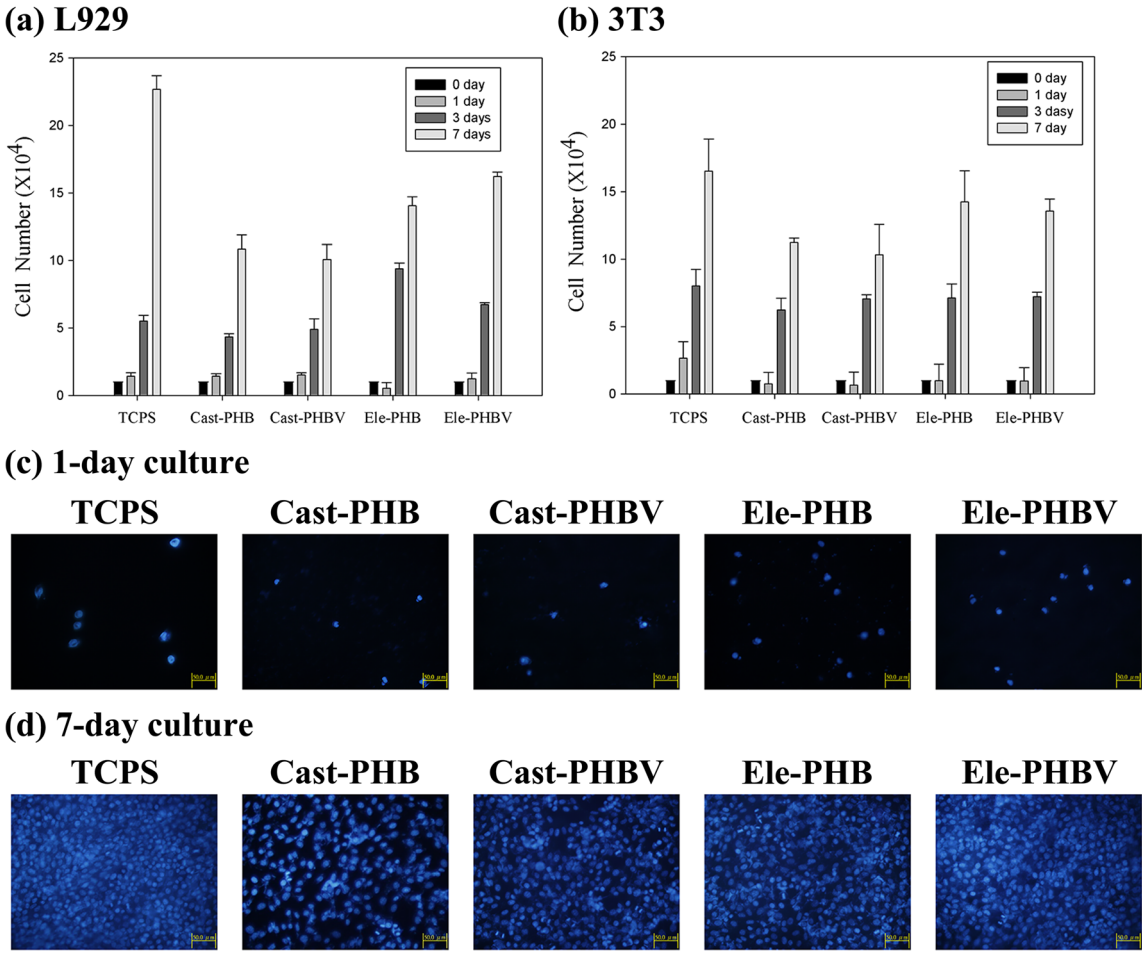

Fig. 2 Cytotoxicity assay of PHB and PHBV. WST-1 assay of (**a**) L929 or (**b**) 3 T3 cell lines seeded on the surface of TCPS, Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV for 1-, 3-, and 7-day culture. The initial cell seeding number is 1×10^4 cells. DAPI staining (blue) of L929 cell line cultured on the surface of TCPS, Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV for (**c**) 1- and (**d**) 7-day. DAPI stained the cell nucleus and measured the cell number. Scale bar is 50 μm

Cell proliferation and morphology of HUVECs and EPCs on PHA films

Based on the result of the cytotoxicity assay in the 3 T3 and L929 cell lines, the potential of Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV films to serve as a scaffold for vascular tissue engineering were evaluated by culturing HUVECs and EPCs (Fig. 3). The WST-1 assay was used to measure the relative proliferation activity of HUVECs and EPCs (Fig. 3a and b). DAPI (for cell nucleus, blue) and F-actin (for cytoskeleton, red) double staining was used to visualize the cell number and morphology (Fig. 3c and d). Within 7 days of culture, the results of the WST-1 assay and DAPI/F-actin staining showed that HUVECs and EPCs can attach and spread on the surface of Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV films. Importantly, HUVECs and EPCs can grow continuously on various types of PHA films. After 7 days of culture, the cell numbers of HUVECs and EPCs on the Ele-PHB and Ele-PHBV films were similar to those on the tissue culture polystyrene (TCPS) and were slightly more than those on Cast-PHB, Cast-PHBV films (no different for EPC numbers). The SEM observation showed that 3 T3 and L929 cell lines can attach and spread on the surface of Ele-PHB and Ele-PHBV films. Interestingly, the morphologies of HUVECs and EPCs were normal and elongated through the direction of the PHA films (Fig. 4). These meant that the Ele-PHB and Ele-PHBV films are more suitable to serve as a scaffold for vascular tissue engineering.

Cell retention evaluation

A closed system with a peristaltic pump and a tube was set up to imitate the vascular flow that occurs in vivo. In the cell retention test, a flow condition of 20 dyne/cm^2 was applied. The loss of HUVECs or EPCs from the surface of PHA films in the early stage was obvious. However, the cell loss rate reduced, and the remaining cell numbers stabilized after 60 mins of exposure to the flow condition (Fig. 5). In addition, EPCs had a stronger ability than HUVECs to attach the surface of PHA films and to resist the shear stress of the flow.

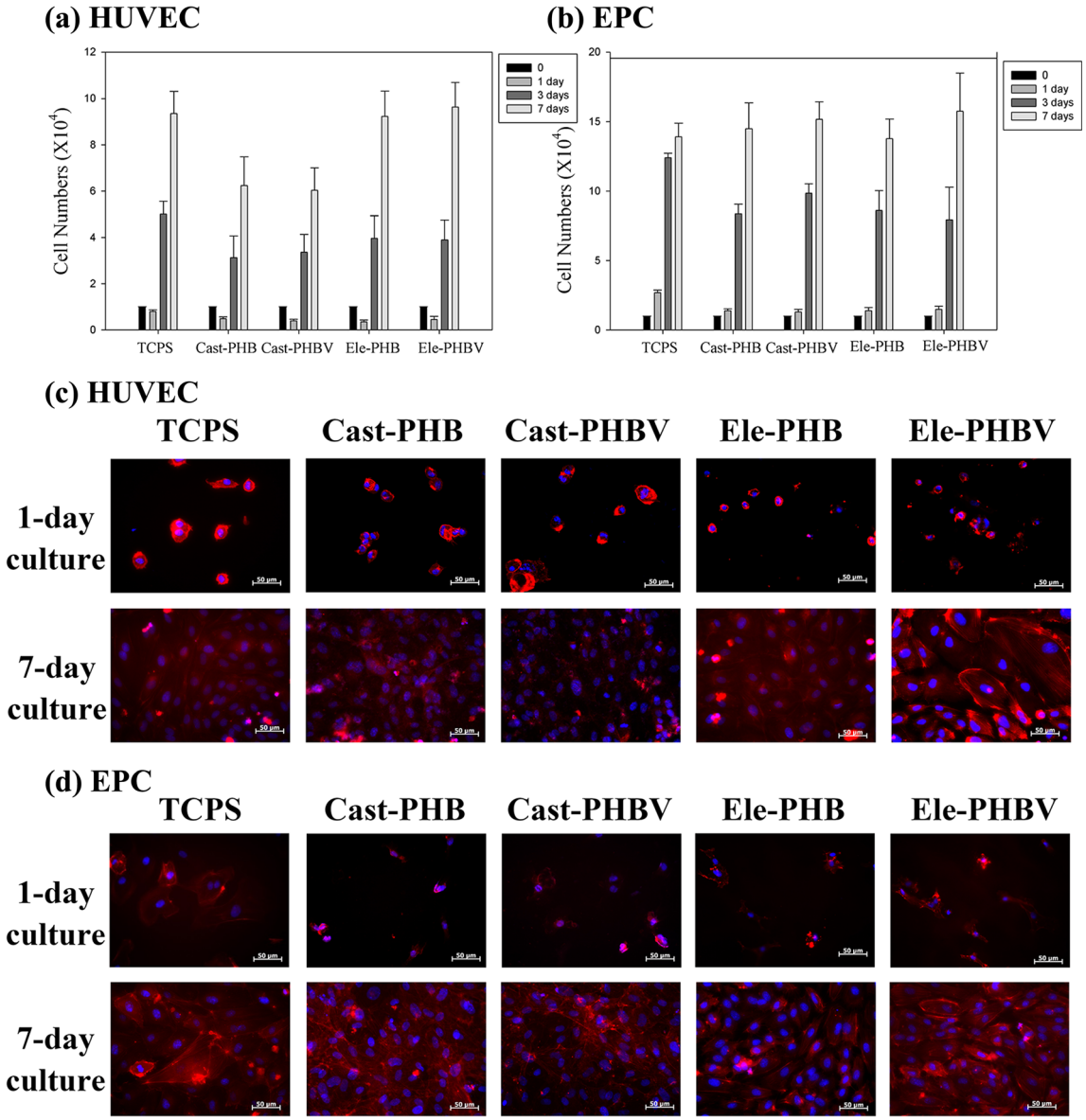

Fig. 3 Cell proliferation assay and staining of primary cells cultured on PHB and PHBV. WST-1 assay of (**a**) HUVECs or (**b**) EPCs seeded on the surface of TCPS, Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV for 1-, 3-, and 7-day culture. The initial cell seeding number is 1×10^4 cells. DAPI (blue) and F-actin (red) double staining of (**c**) HUVECs and (**d**) EPCs line cultured on the surface of TCPS, Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV for 1- and 7-day. DAPI stained cell nucleus and represented as cell number. F-actin stained cytoskeleton and represented as cell structure. Scale bar is 50 μm

This result indicated that the EPCs displayed a great anti-hydraulic pressure ability.

After 180 mins of exposure under the flow condition, the retention rate of EPCs cultured on the Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV were all approximately 75%, higher than those of HUVECs. In the meanwhile, HUVECs cultured on the Ele-PHB and Ele-PHBV (both approximately 69%) had slightly higher cell retention rates than those of the cells cultured on the Cast-PHB and Cast-PHBV (both approximately 62%). These results demonstrated again that the Ele-PHB and Ele-PHBV films benefit the attachment and growth of endothelial-lineage cells, especially for EPCs and are more suitable to serve as a scaffold for vascular tissue engineering.

Anti-coagulation assay

In this study, blood clotting profiles on the various types of PHA films with HUVEC or EPC seeding were determined by the measuring the absorbance at 540 nm (A540) (Fig. 6). Blood clotting resulted in the decrease of A540 due to more platelets being locked into the clot and represented more thrombus formation. This feature can be

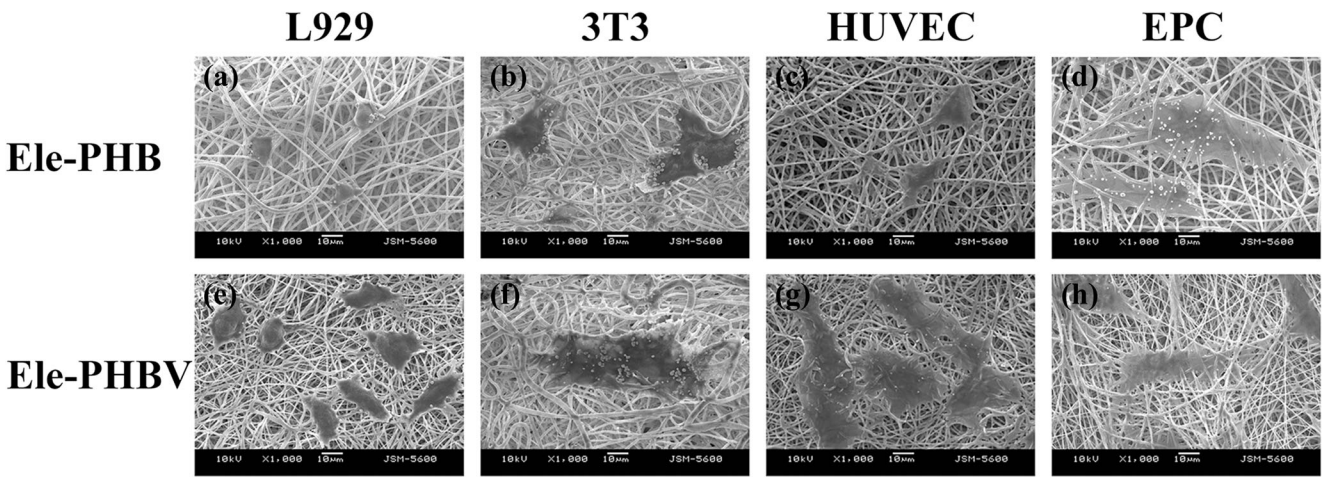

Fig. 4 SEM images of cells cultured on the surface of Ele-PHB and Ele-PHBV for 3 days. The cell morphologies of (**a**, **e**) L929, (**b**, **f**) 3 T3, (**c**, **g**) HUVECs and (**d**, **h**) EPCs on the Ele-PHB and Ele-PHBV, respectively. Scale bar is 10 μm

used to monitor the blood clotting kinetics. The A540 values of 0.5 and 0.1 represented no clot formation at the starting point and the hemoglobin-free condition (distilled water), respectively. The results showed that the A540 values of tubes and non-cell-seeded PHA films (scaffold only) started decreasing at 10–20 min, and the blood samples were difficult to collect after 20 min. However, the A540 values of the HUVEC- and EPC-coated Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV films all stayed at approximately 0.45 for 25 mins. These results demonstrated that the anti-coagulation ability of HUVEC- and EPC-seeded PHA films was superior to that of the non-cell-seeded PHA films. This finding suggested that PHA films coated with HUVECs or EPCs can exhibit antithrombotic properties.

Gene expression of EPCs on PHA films

Not only the number but also the functions of cells on the various types of PHA films were important issue for the application of vascular tissue engineering. Based on the cell retention assay, the results showed that the retention rate of EPCs cultured on the Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV were higher than those of HUVECs (75% vs 62–69%). This result demonstrated that the PHA films are more suitable for the attachment and growth of EPCs. So, we focused on exhibition of EPCs cultured on the PHA films in the following assays.

In this study, relative EPC-specific gene expression of VEGFR-2, vWF, CD31, CD34 and CD133 was analyzed by Q-PCR assays (Fig. 7). The control was the EPC culture on

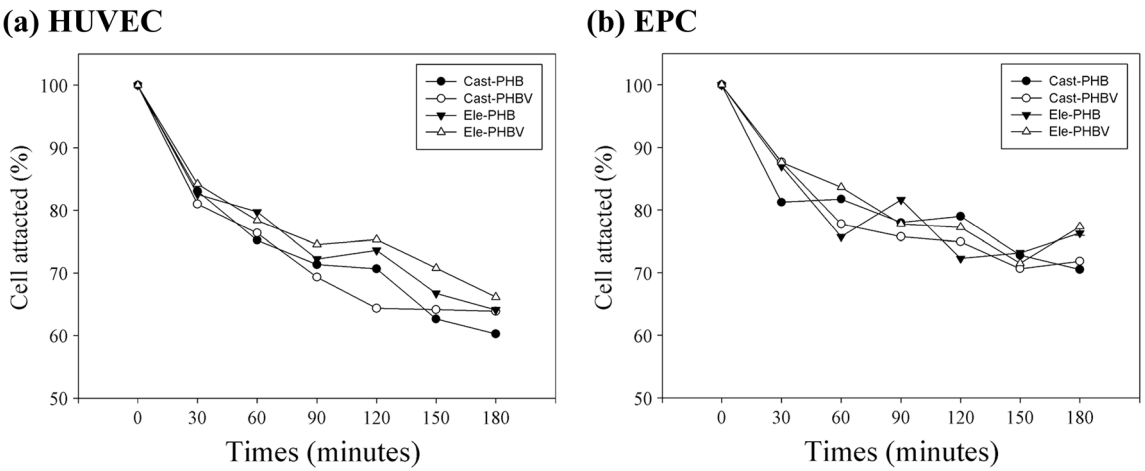

Fig. 5 Cell retention evaluation on various types of PHB and PHBV. **a** HUVECs and (**b**) EPCs cultured on the surface of Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV for 3 days to attach. Then, the cells on scaffolds were exposed continuous steady laminar shear stress (LSS) at 20 dyne/cm^2 which is the peak shear stress in vivo. Subsequently, cells remained to attach on the scaffold surface were determined by WST-1 assay at the indicated time points. Standard deviation of each data is less than 5% (n = 3)

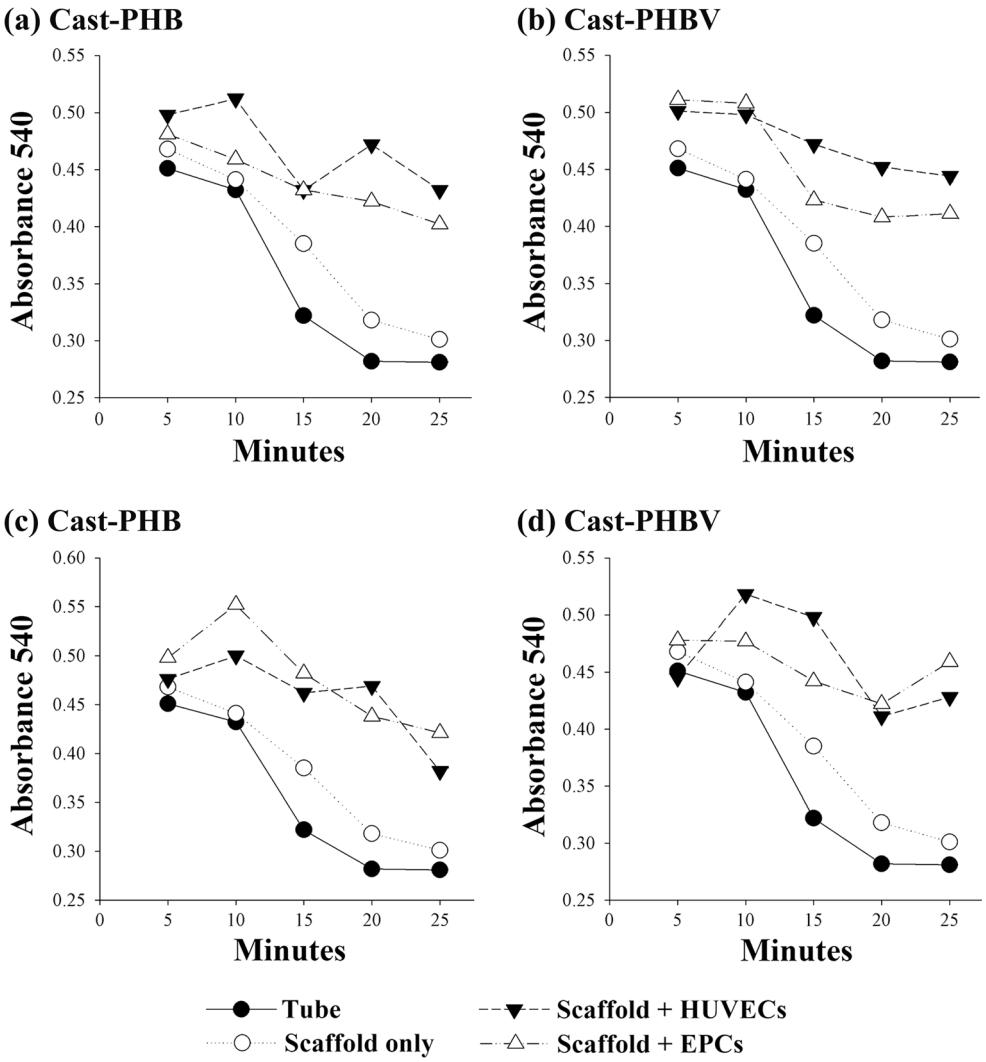

Fig. 6 Anti-coagulation assay on various types of PHB and PHBV with or without cell seeding. The blood coagulation time was measured in (**a**) Cast-PHB, (**b**) Cast-PHBV, (**c**) Ele-PHB and (**d**) Ele-PHBV seeded with or without HUVECs and EPCs by spectrophotometer at wavelength 540 nm absorbance. The anti-clotting time of scaffold seeded with HUVECs or EPCs was significantly longer than in the scaffold without cells ($p < 0.05$). The plastic tube was the control. Standard deviation of each data is less than 5% (n = 3)

the TCPS and set as the unit. The results showed that all EPC-specific gene expression of EPCs cultured on the various types of PHA films (3-dimensional culture) were upregulated (compared to TCPS, 2-dimensional culture) except CD31 expression on Cast-PHB (Fig. 7c). In addition, the endothelial cell-associated gene expression, VEGFR-2, vWF, and CD31 of EPCs cultured on Ele-PHB and Ele-PHBV were higher than those on Cast-PHB and Cast-PHBV. The stem cell-associated gene expression, CD34 and CD133, of EPCs cultured on all PHA films were similar. These results suggest that EPCs can maintain primitive stemness and exhibit correct endothelial cell function on Ele-PHB and Ele-PHBV.

Ac-LDL uptake and UEA-1 lectin binding of EPCs on PHA films

According to the above studies, all the results indicated that Ele-PHB and Ele-PHBV films were excellent scaffolds for vascular tissue engineering. Hence, in addition to the gene expression, other cell functions, including Ac-LDL uptake and UEA-1 lectin binding of EPCs cultured on the Ele-PHB and Ele-PHBV films, were analyzed under a fluorescence microscope. Cholesterol is an essential cellular component of a vessel. LDL is the major carrier of cholesterol and is absorbed by receptor-mediated endocytosis [54]. It is known that UEA-1 lectins bind to glycoproteins such as terminal fucose residues located in the glycocalyx and in the basal membrane of endothelial cells. Hence, lectin binding can be used to recognize the endothelial cell surface. The lectin binding may be caused by ectocytosis, a type of transient plasma membrane opening [55, 56]. The result indicated that the EPCs cultured on different Ele-PHB and Ele-PHBV films can retain the properties of Ac-LDL uptake (red) and UEA-1 lectin binding (green) (Fig. 8). This result suggested that the endocytosis and ectocytosis abilities of EPCs were preserved when EPCs were cultured on the Ele-PHB and Ele-PHBV.

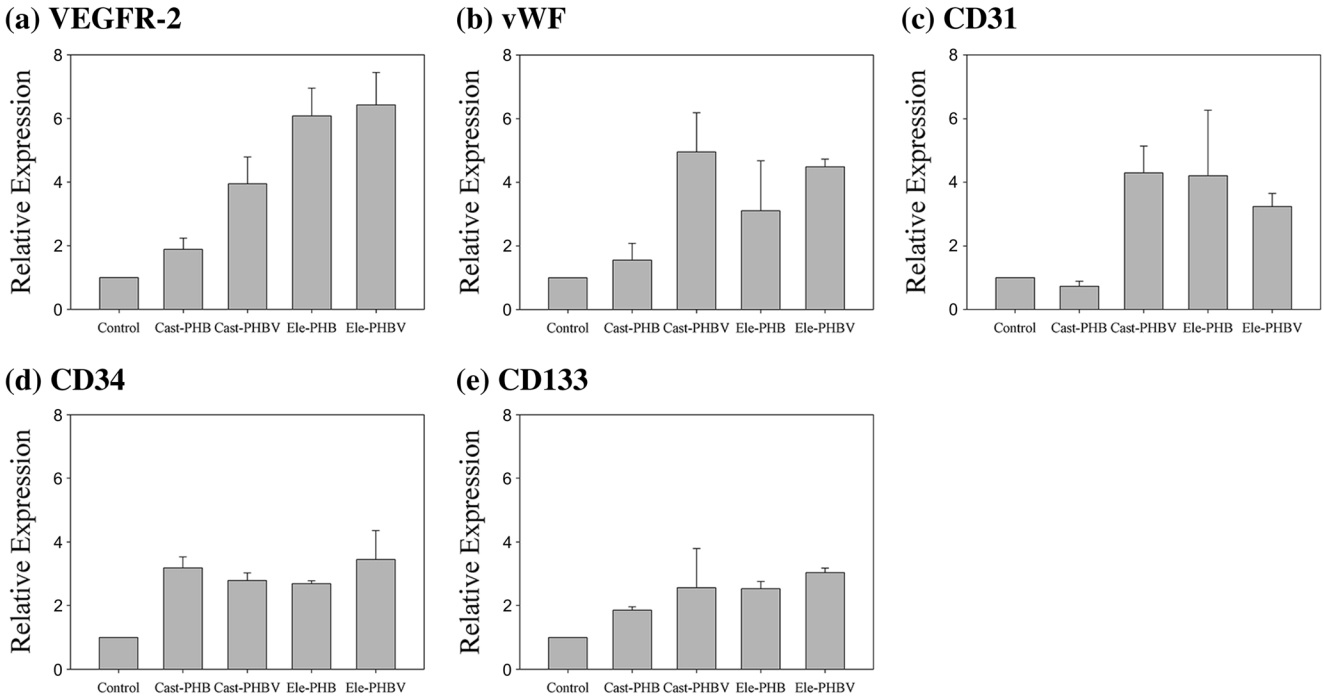

Fig. 7 Real-time PCR analysis of EPCs cultured on various types of PHB and PHBV. Relative EPC-specific gene expression of (**a**) VEGFR-2, (**b**) vWF, (**c**) CD31, (**d**) CD34 and (**e**) CD133 of EPCs after 3 days of culture on the Cast-PHB, Cast-PHBV, Ele-PHB and Ele-PHBV. TCPS were set as control to compare the gene expression

Discussion

PHAs are a superfamily of intracellular polyesters (over 150 kinds of co-monomers have been identified) produced by microorganisms when they uptake excess carbon sources. Importantly, PHAs are biocompatible, biodegradable, and non-toxic and have good thermal and mechanical properties. The purpose of this study was to use the PHAs to fabricate films by solvent casting and electrospinning and explore the application potential to serve as a scaffold for vascular tissue engineering [57–59]. In addition, the microstructure, mechanical and physical properties, cytotoxicity and biocompatibility of various types of PHA films were checked. The growth kinetics, cell viability, adhesion ability, gene expression and functional properties of EPCs and HUVECs cultured on PHB or PHBV films, prepared either by solvent-casting or electrospinning methods, were also analyzed in this study. Tissue engineering primarily combines cells, signals and scaffolds to repair tissues or organs [60]. The scaffold serves as the microenvironment, which is similar to the extracellular matrix

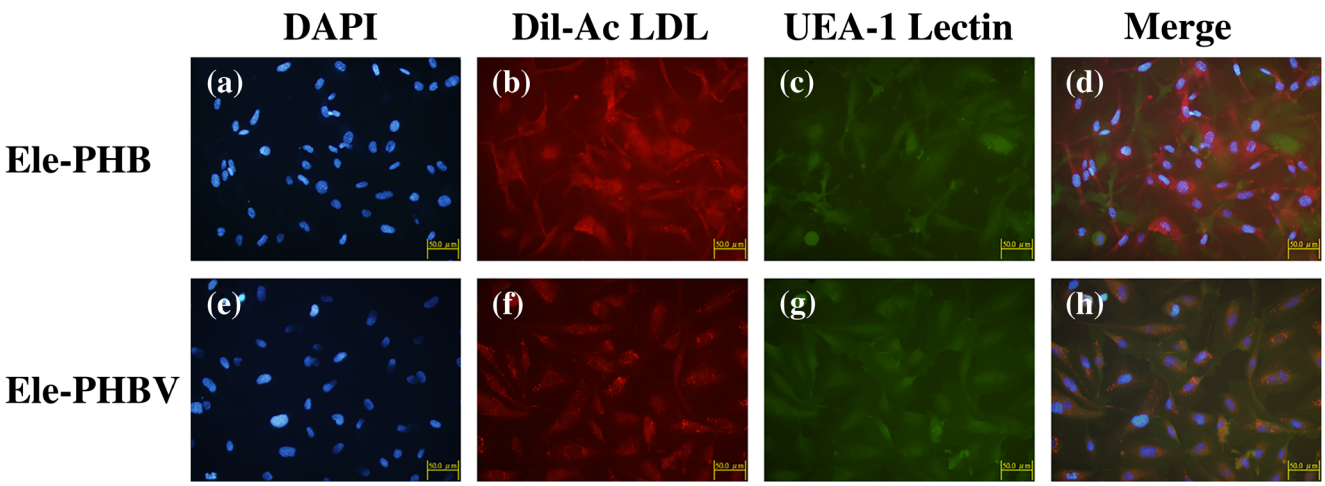

Fig. 8 Functional assays of EPCs cultured on the surface of Ele-PHB and Ele-PHBV. The staining of (**a**, **e**) DAPI, (**b**, **f**) Ac LDL uptakig, (**c**, **g**) UEA-1 Lectin binding and (**d**, **h**) merge of DAPI (blue), Ac LDL (Dil, red) and UEA-1 Lectin (FITC, green) staining of EPCs after 3-day culture on the Ele-PHB and Ele-PHBV, respectively. Scale bar is 50 μm

(ECM), and plays a key role in cell adhesion, proliferation, migration and differentiation [61]; most notably, the electrospun films with nano-porous properties can help the cells grow well [62, 63].

In this study, our results demonstrated that Ele-PHB and Ele-PHBV films had the nano-porous microstructure for cells to grow and suitable mechanical and physical properties for vascular engineering. Ele-PHB and Ele-PHBV also showed less cytotoxicity and high biocompatibility by checking with cultures of 3 T3 and L929 cell lines. Importantly, our results demonstrated that after 7 days of culture of HUVECs and EPCs on Ele-PHB and Ele-PHBV films, the proliferated cell numbers were comparable to cells cultured on TCPS (common cell culture plate). These expanded cells had normal cell morphologies, highly expressed endothelial-associated genes (VEGFR-2, vWF, and CD31) and exhibited correct cell functions (Ac LDL uptake and UEA-1 lectin binding) [64–67]. Ele-PHB and Ele-PHBV films also showed the excellent cell adhesion ability for HUVECs and EPCs under the flow condition (cell retention assay) [68]. Most importantly, Ele-PHB and Ele-PHBV films seeded with HUVECs and EPCs showed an excellent anti-coagulation ability when in contact with blood.

Although the mechanical and physical properties of PHB and PHBV are brittle, their properties could be furtherly improved by copolymerization with other polymers, such as poly(glycerol sebacate) to form elastomer. Elastomer would be more suitable for vascular tissue engineering and will be the next course in our future study. Taken together, based on the results above, PHB and PHBV are suitable bio-based polymers to serve as a scaffold. The combination of electrospinning methods and seeding with HUVECs or EPCs provides a promising technique for vascular tissue engineering. This technique has great potential to treat cardiovascular disease and other clinical conditions in the future.

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Compliance with ethical standards

Conflict of interest The authors indicate no potential conflicts of interest.

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