Short Communication

A poly (saccharide-ester-urethane) scaffold for mammalian cell growth

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Abstract: Chitosan and poly(3-hydroxybutyrate) are non-toxic, biodegradable, and biocompatible polymers extensively used in regenerative medicine. However, it is unknown whether the chemical combination of these polymers can produce a biomaterial that induces an appropriate cellular response *in vitro* in mammalian cells. This study aimed to test the ability of a novel salt-leached polyurethane scaffold of chitosan grafted with poly(3-hydroxybutyrate) to support the growth of three mammalian cell lines of different origin: a) HEK-293 cells, b) i28 mouse myoblasts, and c) human dermal fibroblasts. The viability of the cells was assessed by either evaluation of their capacity to maintain the expression of the green fluorescent protein by adenoviral transduction or by esterase activity and plasma membrane integrity. The results indicated that the three cell lines attached well to the scaffold; however, when i28 cells were induced to differentiate, they did not produce morphologically distinct myofibers, and cell growth ceased. In conclusion, the findings reveal that, altogether, these observations suggest that this foam scaffold supports cell growth and proliferation but may not apply to all cell types. Hence, one crucial question yet to be resolved is a poly (saccharide-ester-urethane) derivative with a nano-topography that elicits a similar cellular response for different biological environments.

Key words: Poly(3-hydroxybutyrate); Scaffold; Polyurethane; Cell-matrix interactions; Engineered matrices.

Introduction

Biomimetic cellular environments can be produced from the synthesis of polyhydroxyalkanoate (PHA) scaffolds (1). Poly(3-hydroxybutyrate) [PHB] is a PHA frequently used for producing new biocompatible materials, with many physicochemical characteristics relevant for biomedical applications, and it has been combined with diverse materials to prepare scaffolds that support cell proliferation (2-4). Due to its excellent biodegradability and biocompatibility properties, chitosan (CS) grafted (g) onto PHB appears to be a viable option to produce scaffolds that maintain cell growth (5, 6). For example, a porous salt-leached poly(saccharide-ester-urethane) composite 3D scaffold using gamma radiation-induced CS-g-PHB supported the growth of pancreatic islets, an endocrine cell type that produces and secretes insulin and glucagon into the bloodstream and is severely damaged in diabetes (7). These results led researchers to examine whether this CS-g-PHB scaf-

fold supports the growth of cells from a different origin.

In this regard, a previous study showed that cells from different origins behave distinctly when grown on salt-leached scaffolds. This is the case of mesenchymal cells that grow well on leached and nanofibrous PHB scaffolds. In contrast, VERO cells, which are isolated from green monkey kidney epithelium, grow better on salt-leached scaffolds (8,9). Thus, it is established that the physical properties of surfaces, derived from the type of technique used to prepare a scaffold, affect cell viability (10,11); nonetheless, there is a significant disparity between different reports, and it is challenging to draw objective conclusions (12-20).

Earlier works have investigated the *in vitro* interaction of mammalian cells with poly (3-hydroxybutyrate-g-vinyl alcohol) polyurethane scaffolds (21) and films of Chitosan/poly-octanoic acid 2-thiophen-3-yl-ethyl ester blends (poly(OTE)) (22). The first study concerns using a grafted PHB as a chain extender to produce a PU but did not include a polysaccharide in the scaffold's

synthesis. The second approach involved evaluating a CS/(poly(OTE)) blend to grow myoblasts. The latter research neither used PHB nor PU formulation in the scaffolds but demonstrated the suitability of the CS-derived blend to maintain self-renewal and myogenic properties compared to neat CS. The above research paved the way for new contributions to a better understanding of the interaction of cells with custom-designed molecular architectures.

In our previous study and others, it has been demonstrated that PHB-based scaffolds properly support cell proliferation (3,10). However, it has yet to be explored whether our recently developed CS-g-PHB PU can support the growth of mammalian cells from different origins (5,7). Thus, this work explored whether three cell lines—HEK-293 cells (a human embryonic kidney cell line), i28 cells (a primary mouse myoblast inducible to differentiate into myofibers), and Human Dermal Fibroblasts (HDF cells)—grow on CS-g-PHB PU.

Materials and Methods

A CS-g-PHB–based scaffold was prepared and characterized as reported earlier (7). Briefly, the prepolymer urethane (polyol conjugated with HDI trimer) reacted with the chain extender (copolymer) and polyethylene glycol (PEG) to yield poly(saccharide-ester-urethane). Sodium acetate (NaAc, J.T. Baker, 149 µm) was included in the mixture (2% (w/w)). After the dough had been pressed, the scaffold was salt-leached using a Soxhlet and water. The route by which CS-g-PHB forms a PU urea scaffold was previously proposed (Fig. 1). First, the zinc 2-ethyl hexanoate (species I) was mixed with HDI isocyanurate (species II) in the presence of PEG, producing a prepolymer (species VI). Then, two chain extenders, the polyol (R1) and graft copolymer (R2) reacted with the prepolymer, finally yielding the grafted CS PU (species VII). We represent the CS-g-PHB PU structure as a complex combination where isocyanate groups can randomly form soft or hard urethane substituted segments (R=R1 or R2).

Before seeding the cells, the CS-g-PHB PU scaffolds were sterilized in an autoclave, then taped onto a Petri culture dish bottom (Nunclon™, cat. # 153066) and sterilized by UV light radiation. Cells were plated on the scaffold and on the dish bottom to compare cell morphology and attachment differences.

HEK-293 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) as previously reported
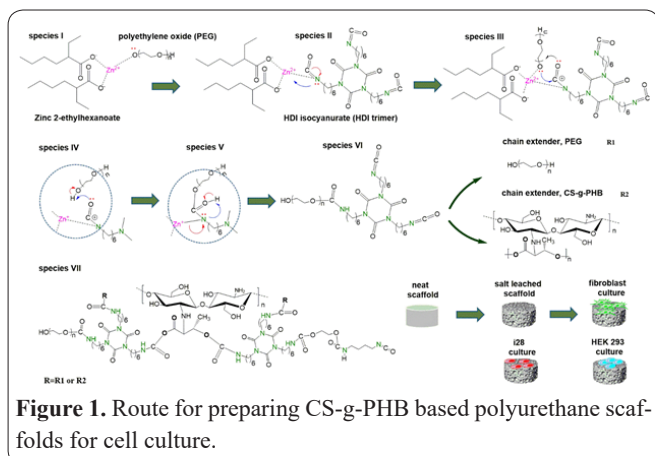
(21), whereas i28 cells were first grown in a propagation medium (F-10 Ham SIGMA®, cat. # N6013) supplemented with 20% fetal calf serum (Gibco®, cat. # 10437-028), 2 mM L-glutamine (Gibco®, cat. # 25030-081), and penicillin-streptomycin 100X (Gibco®, cat. # 15140) and later induced to differentiate by switching to DMEM (Gibco®, cat. # 11995-040) supplemented with 2% horse serum (Gibco®, cat. # 16050130), 2 mM L-glutamine (Gibco®, cat. # 25030-081), penicillin-streptomycin 100X (Gibco®, cat. # 15140). The differentiation medium was replaced every two days for seven to ten days before imaging. To assess the morphology and viability of HEK and i28 cells, these were transduced with an adenovirus that carries the green fluorescent protein (GFP) under the control of the CMV promoter-enhancer (2.5 µL of 3.3 x 10^7 VP/mL in 3.5 mL of medium). HDF cells were grown as described in prior work, and viability was evaluated using the Live/Dead™ viability/cytotoxicity kit (Thermo)(23).

A Zeiss LSM510 confocal microscope was used to assess the morphology and ability of HEK-293 and i28 cells to attach to the CS-g-PHB PU scaffolds. We used a 488 nm line emitted by an argon-ion gas laser for exciting GFP. Rendered images consisted of 60 µm image stacks at 1 µm interval with a 10x objective and 20 µm image stacks at 1 µm interval with a 40X objective.
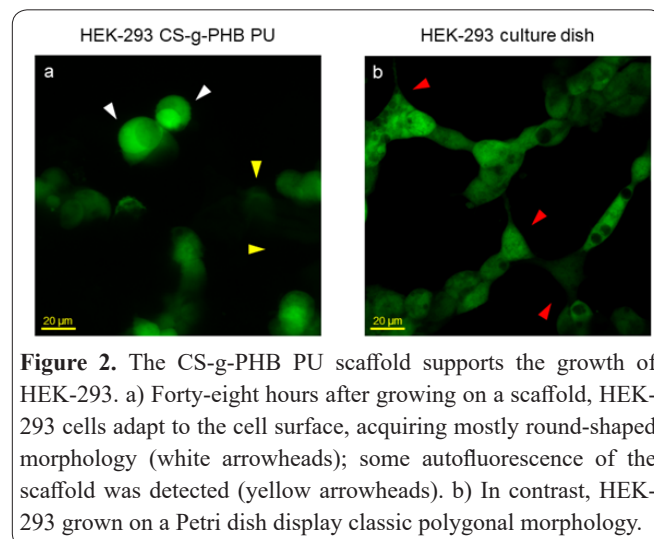
Differences in the size of cells growing on the scaffold and Petri culture dish were determined using ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Graphics were generated by Graph Pad Prism 5.

Results and discussion

Gamma radiation-induced grafting has been recently used for the synthesis of new biomaterials intended for mammalian cell growth (24,25).

From previous results, we had evidence that CS-g-PHB PU scaffolds support the growth of pancreatic islet cells (7). Thus, we intended to expand the applications of this scaffold by exploring its ability to support the growth of different cell types.

First, we examined HEK-293 cells, a fast-growing cell line widely used in cell biology, induced to express GFP via adenoviral transduction. Forty-eight hours after cells were plated on a Petri culture dish or a CS-g-PHB

Figure 1. Route for preparing CS-g-PHB based polyurethane scaffolds for cell culture.

Figure 2. The CS-g-PHB PU scaffold supports the growth of HEK-293. a) Forty-eight hours after growing on a scaffold, HEK-293 cells adapt to the cell surface, acquiring mostly round-shaped morphology (white arrowheads); some autofluorescence of the scaffold was detected (yellow arrowheads). b) In contrast, HEK-293 grown on a Petri dish display classic polygonal morphology.

PU scaffold, they were imaged with a confocal LSM. Inspection of cells grown on CS-g-PHB PU revealed that the cells efficiently attach to the surface; they seem to adapt to the surface by forming small clusters and acquiring a round-shaped morphology (Fig. 2a, white arrowheads). This contrasts with the classic polygonal morphology that cells adopt when grown on the surface of a Petri culture dish (Fig. 2b, red arrowheads). These observations indicate that HEK-293 cells grow well on the scaffold, although their processes do not extend as profusely as when they grow on the culture dish surface. A closer inspection revealed that cells grown on the CS-g-PHB PU scaffold form short lamellipodia in contact with the scaffold surface as if these were mechanical adaptations that attach the cell to the surface (Fig. 3, blue arrowheads, contrasted images). Further imaging is necessary to reveal cellular tension forces that would determine if these lamellipodia are stress fibers— molecular arrays that play an important role in cell adhesion.

Second, we examined the ability of CS-g-PHB PU scaffolds to support the growth and differentiation of i28 cells. i28 is a primary mouse myoblast cell line derived from satellite cells frequently used as an experimental model for the regeneration of skeletal muscle tissue (26). At first, i28 cells were attached to the CS-g-PHB PU surface, forming large cell clusters undistinguishable from those grown on the Petri culture dish (Figs. 3a and b). However, four days after induction of i28 cells differentiation, only a few cells remained attached to the CS-g-PHB PU surface, expanding their cytoplasm and adopting an ovoid shape with short processes (Fig. 4d). The cells were alive since the emission of GFP remained unchanged.
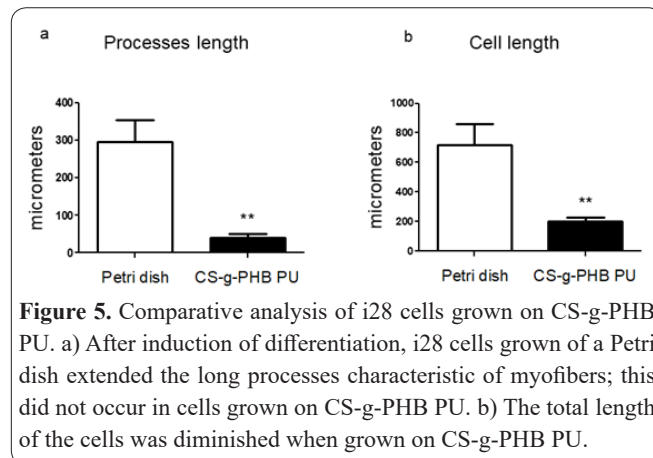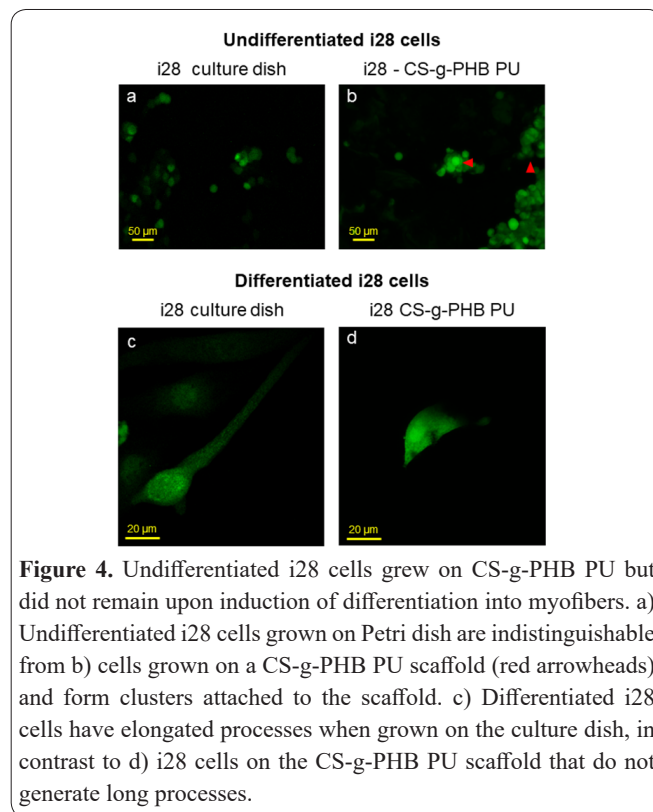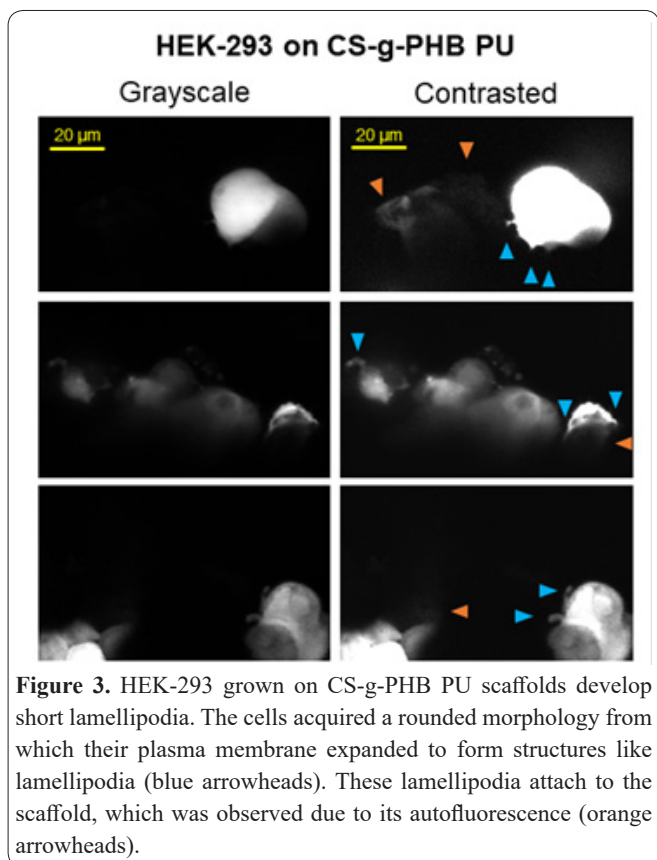
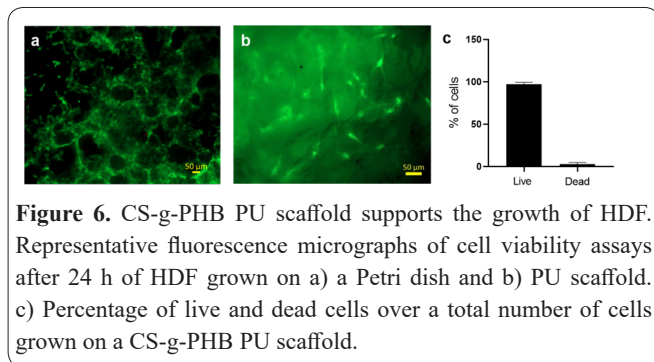In contrast, cells grown on the Petri dish were efficiently induced to differentiate into myofibers (Fig. 4c). These cells extended long processes and divided their nucleus, giving rise to immature myofibers after four days. The length of the processes attached to the scaffold and the total cell length showed significant differences when compared with cells grown on the surface of the Petri culture dish (Fig. 5, t-test, $P < 0.05$). These results show that although CS-g-PHB PU properly supports the growth of immature myoblasts, these do not remain attached to the scaffold's surface during differentiation. It may be possible that changes in the expression of cell adhesion molecules (CAMs) during the process of differentiation into myofibers alter the binding properties of i28 cells to CS-g-PHB PU.

Third, HDF cells were observed under an epifluorescence microscope 24 h after seeding on a CS-g-PHB PU scaffold. At this time, viability was evaluated using a Live/Dead™ viability/cytotoxicity kit which resulted in practically 100% live cells (Figs. 6a and 6b).

Conclusions

This work discloses that CS-g-PHB PU scaffolds efficiently support the growth of HEK-293 cells, undifferentiated myoblasts (i28 cells), and human dermal

Figure 4. Undifferentiated i28 cells grew on CS-g-PHB PU but did not remain upon induction of differentiation into myofibers. a) Undifferentiated i28 cells grown on Petri dish are indistinguishable from b) cells grown on a CS-g-PHB PU scaffold (red arrowheads) and form clusters attached to the scaffold. c) Differentiated i28 cells have elongated processes when grown on the culture dish, in contrast to d) i28 cells on the CS-g-PHB PU scaffold that do not generate long processes.

Figure 3. HEK-293 grown on CS-g-PHB PU scaffolds develop short lamellipodia. The cells acquired a rounded morphology from which their plasma membrane expanded to form structures like lamellipodia (blue arrowheads). These lamellipodia attach to the scaffold, which was observed due to its autofluorescence (orange arrowheads).

Figure 5. Comparative analysis of i28 cells grown on CS-g-PHB PU. a) After induction of differentiation, i28 cells grown of a Petri dish extended the long processes characteristic of myofibers; this did not occur in cells grown on CS-g-PHB PU. b) The total length of the cells was diminished when grown on CS-g-PHB PU.

Figure 6. CS-g-PHB PU scaffold supports the growth of HDF. Representative fluorescence micrographs of cell viability assays after 24 h of HDF grown on a) a Petri dish and b) PU scaffold. c) Percentage of live and dead cells over a total number of cells grown on a CS-g-PHB PU scaffold.

fibroblasts (HDF cells). Our findings also revealed that these cells adapt their morphology to the surface of the scaffold, generating lamellipodia that attach the cell to the scaffold. Unfortunately, CS-g-PHB PU scaffolds did not permit the differentiation of i28 cells into myofibers, a step most necessary if applications for muscle replacement are intended with this cell type.

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The authors have no ethical disclosures to report.

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The authors have no conflict of interest to declare.

Human studies
No human studies were carried out by the authors of this article.

Animal studies
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Interest conflict
There are no conflicts to declare.

Author's contribution
M.G.T.; Data curation, writing original draft. M.B.G.; Investigation, review, editing. G.L.G; Investigation. E.L.; Methodology. O.G.M.; Methodology, investigation. H.C.; Investigation. E.K.R.P.; Investigation. C.P.; Investigation, supervision. A.M.T.; Methodology, conceptualization, supervision.

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