Carbohydrate Polymers 90 (2012) 725–729

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Short communication

Fabrication of chitin/poly(3-hydroxybutyrate-co-3-hydroxyvalerate) hydrogel scaffold

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ARTICLE INFO

Article history:
Received 20 March 2012
Received in revised form 11 May 2012
Accepted 15 May 2012
Available online 23 May 2012

Keywords:
Chitin
Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
Composite
Hydrogel
Scaffold
Skin tissue engineering

ABSTRACT

Regeneration of skin after a large area wound or burn is often difficult without the aid of a scaffold. In this work we developed a 3D macroporous chitin/poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) hydrogel blend by normal solution chemistry and lyophilized it to get the scaffold. The developed scaffold was then characterized and it showed a slow degrading as well as biocompatible nature. The blend hydrogel scaffold showed 67% porosity and the pore size was less than 20 μm. Cytocompatibility of the scaffold was proved by Alamar blue assay using Human Dermal Fibroblast cells (HDF). The blend hydrogel scaffold showed a two fold increase in cell number over control within three days of culture. The chitin/PHBV blend also showed enhanced HDF cell attachment and proliferation. These preliminary results prove its suitability for scaffold based skin tissue regeneration.

1. Introduction

Tissue engineering involves a multitasking effort of material selection, scaffold fabrication and molecular biology for the ultimate goal of replacing a dead or diseased tissue. Skin, the largest organ in the human body, tends to regenerate to a limited extent after a massive injury. A scaffold guided tissue engineering technique would generally outmark the conventional mode of therapy which includes autologous split-thickness skin grafting (Powell & Boyce, 2006). Chitin is a non toxic, completely biodegradable, anti inflammatory, naturally occurring polymer that aids fibroblast proliferation, vascularization (Muzzarelli, 2009). Chitin is widely used in various tissue engineering and drug delivery applications (Jayakumar, Menon, Manzoor, Nair, & Tamura, 2010; Muzzarelli, 2009; Muzzarelli et al., 2007). Chitin is insoluble in most of the organic solvents due to its rigid crystalline structure but dissolves in calcium chloride saturated methanol (Tamura, Furuike, Nair, & Jayakumar, 2011). The main disadvantage of chitin is its high degradability (Jayakumar et al., 2010). PHBV is a microbial derived ester. It has a lesser crystalline structure compared to other polyhydroxy alkanoates and thus shows better degradation behavior. The copolymer degrades to d-3-hydroxy butyric acid which is a constituent of blood (Rezwan, Chen, Blaker, & Boccaccini, 2006). Furthermore there is a positive influence of this degradation product on fibroblast proliferation (Cheng et al., 2006). In addition to being biocompatible it shows piezoelectricity (Farago, Raffin, Pohlmann, Guterres, & Zawadzki, 2008). Thus by blending the two materials we hypothesize to obtain a stable porous scaffold that show enhanced cell proliferation which thus would overcome at least partially the transport limitation which is the major cause of implant failure (Jayakumar et al., 2011). Culture of dermal fibroblasts on macroporous scaffolds developed *via* electrospinning or phase separation confirm cell attachment and proliferation (Guzman, Kirsebom, Solano, Quillaguaman, & Hatti-Kaul, 2011; Ma et al., 2010). In this work we attempt to develop a chitin/PHBV blend *via* a simple technique without affecting the parent polymer properties and showing enhanced stability and controlled degradation.

2. Experimental

2.1. Materials

β-Chitin (85% degree of acetylation) is obtained from Koyo Chemical Co Ltd, Japan. $CaCl_2$, methanol and chloroform (Merck, India). PHBV is obtained in pellet form from Nature Plast, France and it contains 10% by weight 3-hydroxyvalerate. Paraformaldehyde was from Sigma Aldrich, India; glutaraldehyde and hen lysozyme from Fluka. DAPI and actin stain (Texas red phalloidin) were

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http://dx.doi.org/10.1016/j.carbpol.2012.05.041

purchased from Gibco, Invitrogen, India. Human Dermal Fibroblasts and its media were purchased from Promo Cell, India.

2.2. Synthesis of chitin/PHBV scaffold

A chitin solution (3%, w/v) was prepared in methanol saturated with $CaCl_2$. PHBV pellets were dissolved in chloroform (1%, w/v) heated at 60 °C. These two solutions were mixed 1:1 under heating and stirred till a gel formed: the latter was dialyzed against distilled water for 3 days. The hydrogel obtained was frozen and lyophilized to obtain the scaffold.

2.3. Porosity measurement

Liquid displacement method was used to determine the porosity of the scaffolds where a known weight of scaffold (W_d) was immersed in ethanol for 24 h. The wet weight was recorded (W_w). The porosity was calculated using Eq. (A1)

$$\text{Porosity} = \frac{(W_w - W_d) \times 100}{\text{volume of scaffold} \times \rho_{\text{ethanol}}} \quad \text{(A1)}$$

2.4. Biodegradation and swelling studies

In vitro biodegradation was studied by immersing a predetermined weight (W_0) of the scaffold in PBS containing lysozyme (10^4 units/ml) prepared at neutral pH, incubating at 37 °C for different time points and observing their respective dry weights (W_t). The results were compared with the analysis of control chitin scaffold also carried out in the same way. The percentage degradation was calculated using Eq. (A2).

$$\text{Percentage degradation} = \left[\frac{W_0 - W_t}{W_0}\right] \times 100 \quad \text{(A2)}$$

The water uptake or swelling analysis was done at neutral pH and 37 °C. Lyophilized sample was weighed (W_0) and immersed in PBS for predetermined time intervals. The wet weight (W_1) was noted after wiping the excess PBS using a tissue paper. The swelling ratio was calculated using Eq. (A3).

$$\text{Swelling ratio} = \frac{W_1 - W_0}{W_0} \quad \text{(A3)}$$

2.5. Analytical characterization

The morphology of the developed scaffold was studied using a Scanning Electron Microscope (JEOLJSM-6490LA). The lyophilized sample was cut from the interior and gold sputter coated (JEOL, JFC-1600) prior to imaging. The FTIR spectrometer was a Perkin Elmer RX1. Thermogravimetric analysis was carried out using TG/DTA instrument (EXSTAR-SII TG-DTA 6200) where 3 mg of sample was subjected to heating from 25 to 500 °C.

2.6. Serum protein adsorption study

Sterile scaffolds of equal size and shape were placed in 96 well plate and incubated with Fibroblast growth media (FGM) + 10% FBS at 37 °C for predetermined time points. The scaffolds were then washed thrice with PBS and incubated with elution buffer. Total protein was quantified using bicinchoninic acid (BCA) assay as in Sudheesh Kumar et al. (2011). Briefly BCA reagent was added to each well and incubated at 37 °C. The absorbance was measured at 562 nm. Scaffold incubated in serum free media were used as control.

2.7. Cell viability study

Cell viability on chitin/PHBV scaffolds was evaluated using Alamar blue assay. HDF cells were seeded at a density of 7500 cells/scaffold in a 96 well plate and incubated at different time points. Viability was analyzed by replacing media after 12 and 72 h with 200 μl fibroblast growth medium containing 10% Alamar solution. After required incubation the optical density was measured at 570 nm and 600 nm as the reference wavelength in a microplate reader (Biotek PowerWave XS, USA). Cell number corresponding to the optical density was analyzed from the standard curve plotted.

2.8. Cell attachment and proliferation study

HDF cells were seeded at a density of 15,000 cells per scaffold in a 24 well plate and cultured for predetermined time period (6 h and 24 h). The scaffolds were then washed and fixed with 0.25% of glutaraldehyde. The scaffolds were dehydrated and dried in room temperature. Cell attachment on to scaffolds were visualized in SEM imaging. Fluorescence imaging was done after culturing cells on the scaffold for 24 and 48 h. After the predetermined time

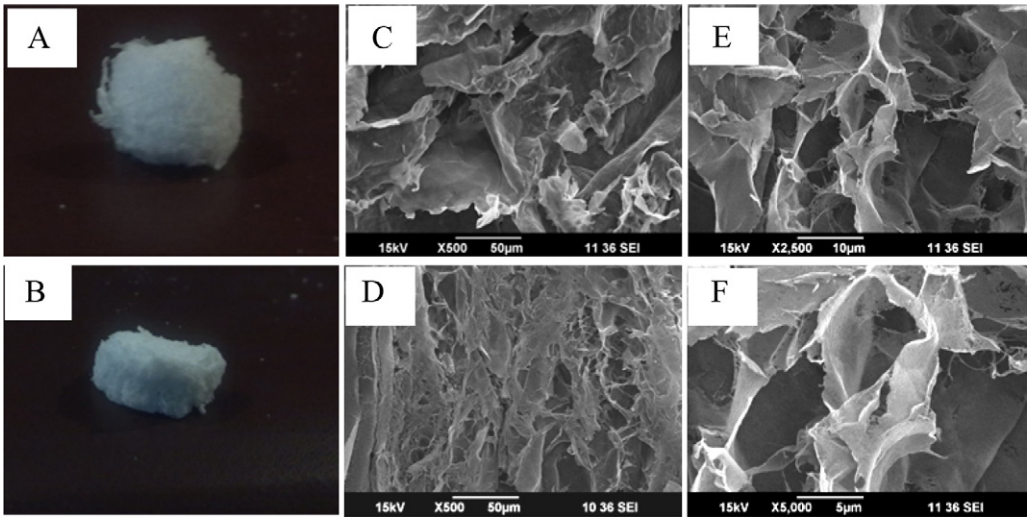

Fig. 1. Photograph of lyophilized chitin control scaffold (A) and chitin PHBV (B) and their SEM images (C), (D). SEM images of (B) at higher magnification (E), (F).

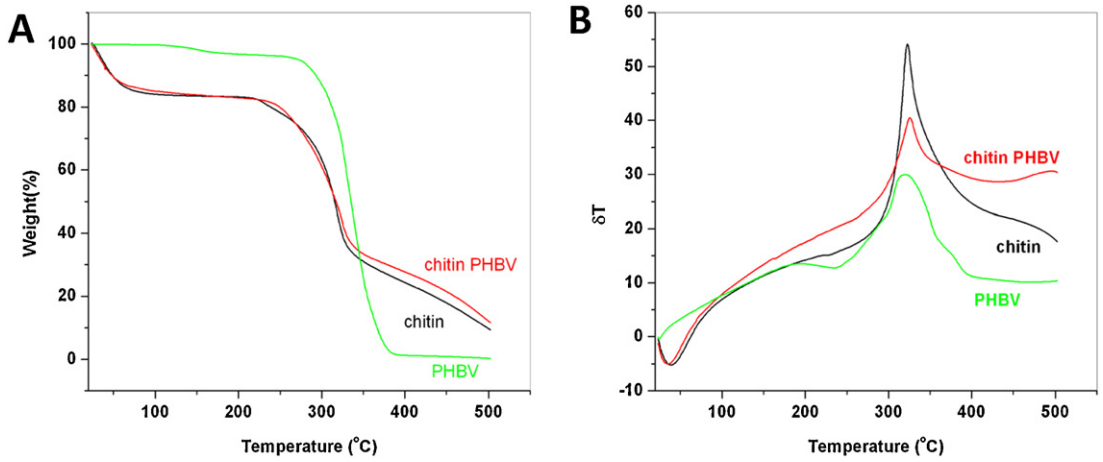

Fig. 2. TGA (B) and DTA of the chitin/PHBV scaffold and controls.

points the scaffolds were washed with PBS twice and fixed with 4% PFA. Cells were permeabilized using 0.5% Triton X and stained for nucleus using DAPI and viewed under fluorescent microscope (Olympus-BX-51).

2.9. Statistics

Results are expressed as mean ± SD of $n \geq 3$. Statistical analysis was done using Student's t test with a confidence level of 95%. The error bars denote ±SD.

3. Results and discussion

The SEM images, in Fig. 1C–F, show a porous structure with pore size ranging from 5 to 20 μm when compared to the control scaffold whose pore size were much larger. The average porosity of the chitin/PHBV blend hydrogel was 67% and that of 61% for the control indicating no significant change in porosity. This indicates that the pore size could be tuned down to lower microns without changing the overall porosity. This would greatly increase the surface area of the scaffold for effective cell attachment. Reports also shows that keratinocytes grow effectively in scaffolds with pore size as low as 20–50 μm (Wang et al., 2006). Moreover the developed scaffold was not brittle as with the case of most of the 2D films developed from PHBV (Ji, Li, & Chen, 2008).

The characteristic band of PHBV lies at 1735 cm^{-1} which indicates the C=O ester group stretch. The presence of this band in the blend confirms the incorporation of PHBV in the composite. The chitin specific bands were also explicated in the FTIR of the composite. The bands include the amide I region at 1645 cm^{-1}, amide II region at 1567 cm^{-1}. The sharp band at 1384 cm^{-1} depicts $-CH_3$

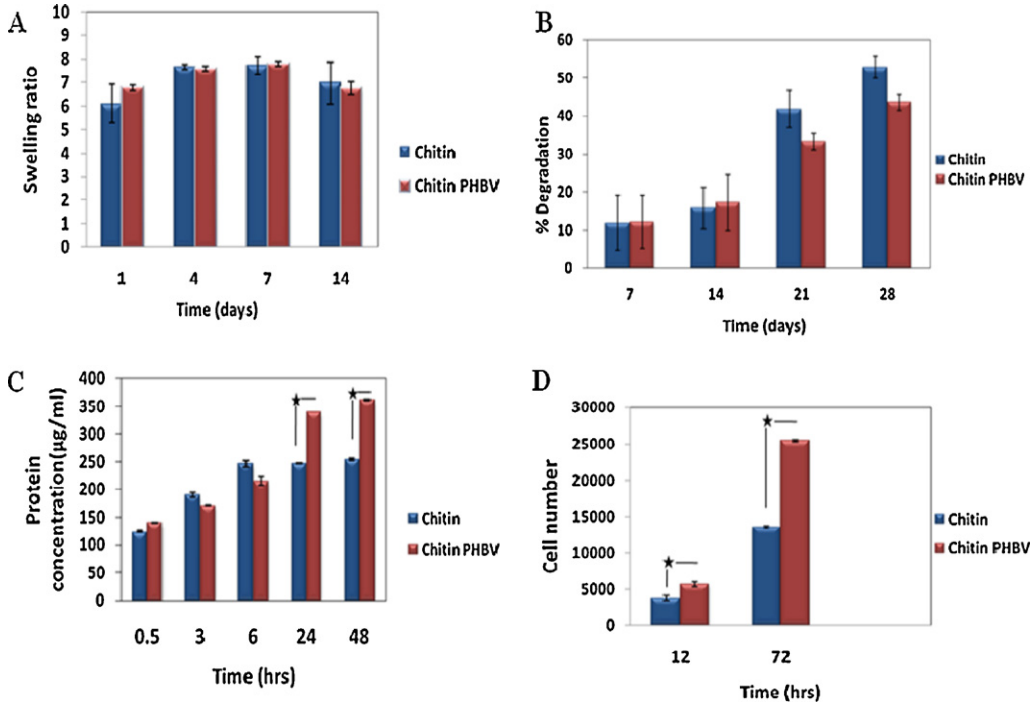

Fig. 3. Swelling study (A) of chitin (blue) and chitin PHBV (red) in PBS. Graph showing degradation (B) in PBS containing lysozyme. Protein adsorption (C) at different time intervals and cell viability (D) determined using Alamar blue assay on Human Dermal Fibroblasts. $p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

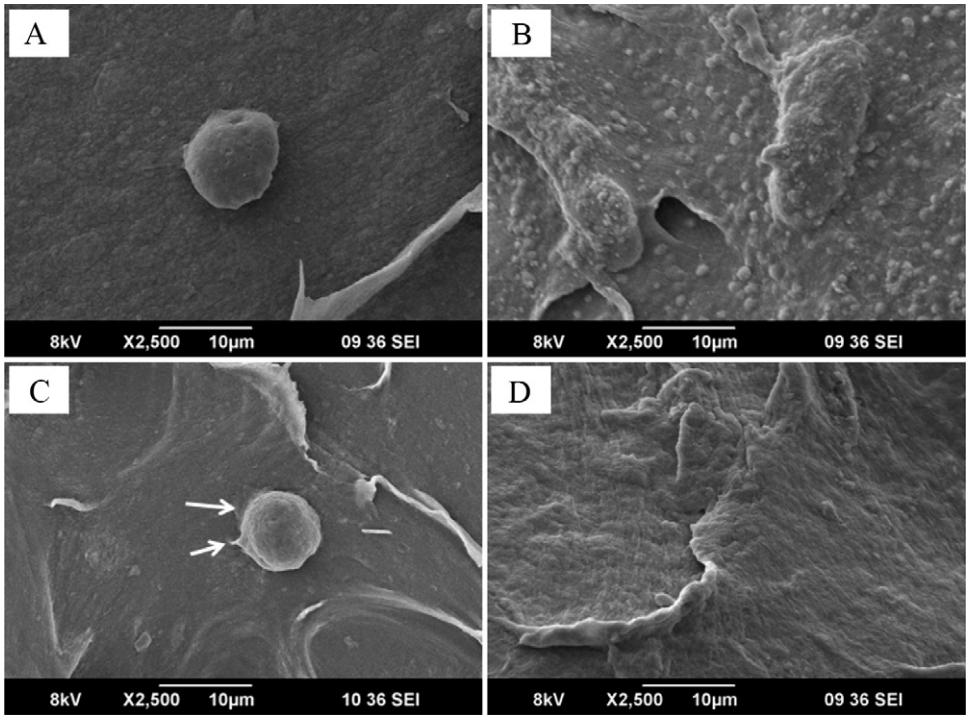

Fig. 4. SEM images showing initial and complete dermal fibroblast attachment on chitin (A, B) and chitin PHBV (C, D). Initial cell attachment at 4 h shows distinguished filopodial extensions in chitin PHBV (white arrows) and a completely spread morphology compared to chitin after 72 h of culture.

symmetrical deformation. The existence of β (1–4) glycosidic bond was confirmed by the band at 1069 cm $^{-1}$.

Fig. 2A and B shows the thermogram of chitin, PHBV and chitin/PHBV. Both chitin and chitin/PHBV shows a two step degradation process. Evaporation of water was found at the range of 50–100 °C and the second at 250–300 °C indicates the degradation of the polymer to its monomer units. PHBV shows an endothermic melting peak at around 200–250 °C and decomposition at the range of 300–350 °C.

Fig. 3A shows a controlled swelling when compared to the control chitin scaffold. Biodegradation study (Fig. 3B) shows a lagging degradation of the blend compared to the control. This probably

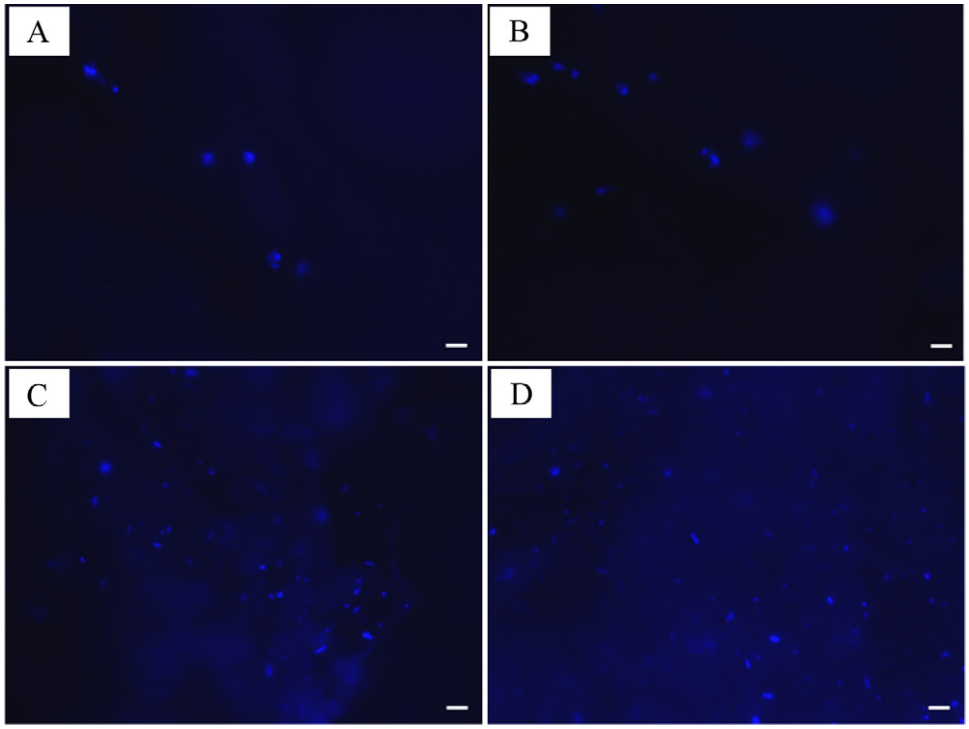

Fig. 5. Fluorescent imaging of cells (nucleus stained blue using DAPI) attached within the chitin scaffold at 24 h (A) and 48 h (B) and in chitin PHBV scaffold at 24 h (C) and 48 h (D). Scale bar denotes 20 μm.

would attribute to the stronger hydrogen bonding between the chitin and PHBV. The degradation of chitin occurs in the presence of lysozyme since it can specifically cleave the glycosidic linkages of chitin, producing N-acetyl-D-glucosamine and the D-glucosamine monomers, which will further enhance the bioactivity of the materials (Sudheesh Kumar et al., 2011).

Fig. 3C shows a continuous and steady increase in serum protein adsorption with time for the blend when compared to the control which attains saturation within 6 h of incubation time. The steady increase in adsorbed protein might be due to the addition of PHBV as there would be a slight increase in hydrophobicity of the scaffold compared to the control chitin (Ali et al., 2011; Dufresne, Dupeyre, & Paillet, 2003).

A significant increase in cell number is evident from Fig. 3D indicating the biocompatibility of the developed blend. Alamar blue assay explicit this result. There was significant increase in cell attachment initially which later showed a marked increase in number after 3 days of culture. The initial cell attachment (4 h) shows a round morphology in the control scaffold; but in the blend slight filopodial extension could be seen (Fig. 4A and C). By 72 h elongated cells spreading to a vast area in the blend could be seen; whereas, cells in the control sample showed lesser extend of spreading (Fig. 4B and D). DAPI staining of the nucleus shows a considerable increase in cell number in blend after 48 h which indicates proliferation (Fig. 5A–D).Initial number was also significantly higher for the blend than the control.

4. Conclusions

This work reports a simple technique to develop a macroporous chitin/PHBV composite scaffold. The hydrogel blend scaffold was synthesized and characterized using SEM, FTIR and TG/DTA. The scaffold showed optimum porosity, controlled swelling and biodegradation. The macroporous and bioactive nature of the scaffold aided HDF cell attachment and proliferation. The marked improvement in cell attachment and spreading on the hydrogel scaffold greater that on reported 2D films (Ji et al., 2008) of the same makes the developed chitin/PHBV blend hydrogel scaffold a promising candidate for skin tissue engineering.

Acknowledgements

One of the authors R. Jayakumar is grateful to the Department of Biotechnology (DBT), India, for providing fund under the scheme of Nanoscience and Nanotechnology Program (Ref. No. BT/PR 13585/NNT/28/474/2010). This work was also partially supported by Nanomission, Department of Science and Technology (DST), Government of India.

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