Fibres de polyhydroxyalkanoate (PHBV) obtenues par une méthode de filage humide : bonne cytocompatibilité in vitro mais absence de biocompatibilité in vivo en site osseux

Cristinel Nicolae Degeratu, Guillaume Mabilleau, Eric Aguado, Romain Mallet, Daniel Chappard, Corneliu Cincu, Izabella-Cristina Stancu

▶ **To cite this version:**

Cristinel Nicolae Degeratu, Guillaume Mabilleau, Eric Aguado, Romain Mallet, Daniel Chappard, et al.. Fibres de polyhydroxyalkanoate (PHBV) obtenues par une méthode de filage humide : bonne cytocompatibilité in vitro mais absence de biocompatibilité in vivo en site osseux. Morphologie, 2019, 103 (341-2), pp.94-102. 10.1016/j.morpho.2019.02.003 . hal-02869320

HAL Id: hal-02869320

https://univ-angers.hal.science/hal-02869320v1

Submitted on 25 Oct 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

Version of Record: https://www.sciencedirect.com/science/article/pii/S1286011519300244
Manuscript_18d917b2d5b7c6c94ad66a44e8edd4d3

Polyhydroxyalkanoate (PHBV) fibers obtained by a wet spinning method: good *in vitro* cytocompatibility but absence of *in vivo* biocompatibility when used as a bone graft

Titre français : **Fibres de polyhydroxyalkanoate (PHBV) obtenues par une méthode de filage humide : bonne cytocompatibilité *in vitro* mais absence de biocompatibilité *in vivo* en site osseux.**

C.N. Degeratu[a,b], G. Mabilleau[b,c], E. Aguado [d], R. Mallet[b,c], D. Chappard[b,c*], C. Cincu[a], I.C. Stancu[a]

[a] *University Politehnica of Bucharest, Faculty of Applied Chemistry and Materials Science, Department of Bioresources and Polymer Science, 1-7 Gh Polizu, 011061, district 1, Bucharest, Romania*

[b] *Groupe Etudes Remodelage Osseux et bioMatériaux, GEROM, EA-4658, SFR-4208, Univ-Angers, IRIS-IBS Institut de Biologie en Santé, CHU-Angers, 49933 ANGERS-FRANCE.*

[c] *Service Commun d'Imagerie et Analyses Microscopiques, SCIAM, SFR-4208, Univ-Angers, IRIS-IBS Institut de Biologie en Santé, CHU d'Angers, 49933 ANGERS Cedex -FRANCE.*

[d] *ONIRIS, Ecole Vétérinaire de Nantes, 44307 Nantes Cedex 3 France.*

Address for correspondence:

* Daniel CHAPPARD, M.D., Ph.D. GEROM - LHEA IRIS-IBS Institut de Biologie en Santé, Univ-Angers CHU d'Angers, 49933 ANGERS Cedex -FRANCE	**Tel:** (33) 244 68 83 43 **e-mail:** daniel.chappard@univ-angers.fr

© 2019 published by Elsevier. This manuscript is made available under the CC BY NC user license
https://creativecommons.org/licenses/by-nc/4.0/

Summary

Polyhydroxyalkanoates (PHAs) are biomaterials widely investigated for tissue engineering applications. In this regard, we describe a method to prepare fibers of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) by a wet-spinning technique. Polymer fibers were used to test the cytocompatibility of the material *in vitro*. We have investigated their behavior *in vitro* in presence of the osteoblast-like (SaOs2) and macrophage (J774.2) cell lines. The PHBV fibers used were 100-200 μm in diameter and offered a large surface for cells adhesion, similar to that they encounter when apposed onto a bone trabeculae. The fiber surface possessed a suitable roughness, a factor known to favor the adherence of cells, particularly osteoblasts. PHBV fibers were degraded *in vitro* by J774.2 cells as erosion pits were observable by transmission electron microscopy. The fibers were also colonisable by SaOs2 cells, which can spread and develop onto their surface. However, despite this good cytocompatibility observed *in vitro*, implantation in a bone defect drilled in rabbit femoral condyles showed that the material was only biotolerated without any sign of osteoconduction or degradation *in vivo*. We can conclude that PHBV is cytocompatible but is not suitable to be used as a bone graft as it does not favor osteoconduction and is not resorbed by bone marrow macrophages.

204 words

Short running title: Polyhydroxybutyrate fibers, cytocompatibility and biocompatibility in bone.

Key words: polyhydroxybutyrate; cytocompatibility; biopolymers; biomaterials; bone graft.

Résumé

Les polyhydroxyalcanoates (PHAs) sont des biomatériaux largement étudiés pour des applications d'ingénierie tissulaire. À cet égard, nous décrivons un procédé de préparation de fibres de poly (3-hydroxybutyrate-co-3-hydroxyvalérate) (PHBV) par une technique de filage humide. Des fibres de polymère ont été utilisées pour tester la cytocompatibilité du matériau *in vitro*. Nous avons étudié leur comportement *in vitro* en présence de lignées cellulaires ostéoblastiques (SaOs2) et macrophagiques (J774.2). Les fibres de PHBV utilisées avaient un diamètre de 100 à 200 µm et offraient une grande surface d'adhésion pour les cellules, semblable celle qu'elles rencontrent lorsqu'elles sont fixées sur une travée osseuse. La surface de la fibre possédait une rugosité appropriée, facteur connu pour favoriser l'adhérence cellulaire, en particulier celle des ostéoblastes. Les fibres de PHBV ont été dégradées *in vitro* par des cellules J774.2 et des zones d'érosion ont été observées par microscopie électronique à transmission. Les fibres étaient également colonisables par les cellules SaOs2, qui peuvaient se propager et se multiplier à leur surface. Cependant, une implantation dans un défaut osseux créé dans le condyle fémoral chez des lapins a montré que le matériau était uniquement biotoléré sans aucun signe d'ostéoconduction ni de dégradation. Nous avons constaté que le PHBV est cytocompatible *in vitro*, mais qu'il ne convient pas pour une greffe osseuse car il n'est pas ostéoconducteur et il n'est pas degradé par les macrophages de la moelle osseuse *in vivo*.

Introduction

Polymers represent a suitable alternative to prepare bone grafts. They can be prepared in large amounts, at low cost and can be polymerized in various forms. They can easily be cut by the surgeon to the desired size in the operating room. Some polymers allow the entrapment of biologically active molecules such as drugs, growth factors or proteins. Resorbable polymers such as poly(lactic acid) (pLA) or poly(glycolic acid) (pGA) have been extensively documented in the literature. However, they are degraded by progressive hydrolysis in the body microenvironment and release acids (lactic and glycolic acid) that accumulate locally and favor inflammation during months or years after implantation [1]. In addition, *in vitro* studies have found a poor adherence of bone cells associated with a reduced cell growth [2]. pLA and pGA have been used to prepare sutures or bone pins for internal fixation of fractured long bones.

An ideal biomaterial should be osteoconductive (*i.e.* favor the bone ingrowth from the vicinal osteoprogenitor cells) and should be resorbed progressively. These polymers must therefore fulfill several conditions: suitable mechanical properties, interconnected porosity, excellent cyto and biocompatibility.

Biopolymers such as polyisoprenoids, polyhydroxyalkanoates (PHAs), glycosaminoglycanes, polypeptides, polysaccharides, polynucleotides are present naturally in our environment. The general formula of PHAs is presented Fig. 1. Polyhydroxybutyrate (PHB) is a member of this group, in which R=CH_3 and m=1 [3-7]. Discovered in the mid 20's by Lemoigne [8], PHB has stayed in the shadow more than 50 years, during which only a few studies were published. PHB, like the majority of PHAs, is produced as carbon storage source by microorganisms, in certain stress conditions and imbalanced nutrient supply [9]. Microbial biosynthesis of PHB starts by

polymerizing soluble intermediates into insoluble granular ones in order to preserve cells osmotic state and to prevent leakage of the nutrient [7,10]. The accumulation of discrete PHAs granules in microorganisms is up to 90% of the cell weight (in dried state) [11]. Until present, more than 150 hydroxyalkanoate units have been isolated; any of these units can lead to a PHA, and any combination of two units can form a copolymer [12]. The differences between PHAs depend on R-pendant groups and on the number *m* of CH_2 units [5,7,9,10,13,14]. Due to their properties, poly(3-hydroxyalkanoates) have attracted a large interest of industrial production especially in applications which require a biodegradable and biocompatible thermoplastic polymer [15,16]. In particular, poly(3-hydroxybutyrate) (P3HB) and related copolymers with 3-hydroxyvalerate have attracted considerable interest for being used as biomaterials [5,9,10,17-19].

For a comprehensive understanding of the polymers biocompatibility, we should consider the cell behavior in presence of the degradation products, including oligomers and monomers and not just the implant itself. 3-hydroxybutyric acid (3HB), the most common PHA degradation product, has been reported to have various therapeutic effects [20-23]. It was also shown that oligomers of 3HB (dimer and trimer) are rapidly degraded to monomers in rat and human tissues, serving as an energy substrate for injured patients [24].

The studies conducted with biomaterials in tissue engineering applications include both natural and synthetic biodegradable polymers [25]. Biodegradable polymers are used as support for osteoconduction which allows osteoblast (bone forming cells) to form new bone on their surface together with resorption [26]. Bone tissue engineering can provide solutions using three-dimensional (3D) porous scaffolds for generating new bone tissue

[27]. Biomaterials are therefore used to fill localized bone loss in orthopedic, maxillo-facial surgery and odontology. The biocompatibility of a material depends on its chemical composition, but also on other factors such as its amount, microarchitecture, degree of anisotropy, porosity (connected or not).

In the present study, polyhydroxyalkanoates fibers based on poly(3-hydroxybutyrate-co-3-hydroxyvalerate) were prepared by a wet-spinning method. Cytocompatibility of the fibers was evaluated *in vitro* with osteoblast and macrophage-like cells. The biocompatibility was evaluated *in vivo* after implantation in the femoral condyle of four rabbits.

Material and methods

Fibers preparation

Polyhydroxybutyrate- 98%/polyhydroxyvalerate 2% (PHBV) - biopolymer powder was purchased from Goodfellow SARL (France) and used without any purification. PHBV fibers were prepared by a coagulation-precipitation in a coagulation non-solvent bath method (the wet-spinning method) using a 20% (w/v) chloroform solution. Briefly, the PHBV chloroform solution was obtained by dissolving 4g of polymer powder in 20 mL chloroform, under stirring at 70°C. Five mL of polymer solution were cooled at room temperature and extruded from a 10 mL commercial syringe into a 1 L ethanol bath (the diameter of the bath was much lower than its height), resulting in the formation of individual PHBV fibers. The extrusion rate was 0.2 mL/min. After 5 minutes every single obtained fiber was collected and let dry - if not used immediately - for 48 h in the air at

room temperature. Others substances were bought from Sigma-Aldrich Chemicals and used without any purification.

Fibers scaffolds preparation

2D and 3D scaffolds were prepared. The 2D scaffolds were made by sticking fibers together as a non-woven network: samples of 5 cm long fiber were laid on a piece of filter paper to obtain a network. Afterwards, the obtained scaffold was covered by another piece of filter paper. At the end, the sandwich formed by the filter paper and fibers scaffold was wet with a 1:1 mixture of acetone and methylene chloride and pressed with a 1 kg weight. The pressure was maintained overnight.

The 3D scaffolds were prepared from freshly fabricated fibers. Briefly, a fibrous 3D scaffold (3 cm long) was prepared from a 6 m long fiber by folding it to the desired dimension. During this operation, the polymer fiber was maintained wet by introducing it from time to time into the ethanol bath. At the end, the 3 cm long folded fibers were rolled up and introduced in a 1 mL syringe (4 mm in diameter), to facilitate their implantation in a bone defect and then let to dry at room temperature, for 48 h. After the complete drying, a 3D scaffold was obtained. All operations were conducted at room temperature. Fibers were controlled under fluorescence optical microscopy.

Cell culture

Two cell lines were used: osteoblast-like cells – SaOs2 (derived from a rat osteosarcoma) and murine macrophage cell line – J774.2 (European Collection of Cell Culture, Salisbury, Wiltshire, England). 3D dried scaffolds were transferred in 6-well culture vials and sterilized for 24 hours under UV radiations 260 nm wavelength. Dulbecco's Modified Eagle's Medium (DMEM) enriched with fetal calf serum 10%, penicillin (100

UI/ml) and streptomycin (100 mg/ml) were added. Fiber scaffolds were equilibrated at 37°C for 1 h and the medium was discarded. One milliliter of DMEM containing 10^5 cells/mL was added and the cells were cultured for 1, 3 and 7 days for SaOs2 and 1, 3, 7, 14 and 21 day for J774.2 (medium changed every two days). During the culture period, samples were kept in a humidified oven under 5% CO_2 and examined with an inverted optical microscope.

Cell viability assay

In order to test cytoxicity of the polymer fibers, an exclusion test with trypan blue and two cell lines, was used. Briefly, the test protocol can be summarized as follows: a cell suspension (10^5 cell/mL) was incubated in presence of 5 mg of PHBV fibers with trypan blue 0.04% and transferred into a hemocytometer. Living (clear) and dead (blue) cells were counted under light microscope examination and the percentage of dead cells was determined for each condition, according to the following formula:

% of dead cells = 100 x (number of dead cells) / (number of dead cells + number of living cells).

For each cell line and incubation period the experiment was conducted in triplicate.

Scanning electron microscopy (SEM)

Cell adherence was investigated by SEM. Prior to imaging, samples were processed as previously described [28]. After incubation with cell lines (SaOs2 and J774), PHBV fibers were rinsed in phosphate buffer 0.1M, pH7.4 and fixed in glutaraldehyde 2.5% (w:v) in phosphate buffer prior to be rinsed in phosphate buffer. Fibers were post-fixed in osmium tetroxide (1% dissolved in distilled water) for 45 min and rapidly rinsed with distilled water. They were then dehydrated using a concentration gradient of ethanol and

desiccated with hexamethyldisilazane (HMDS). HMDS was let to evaporate overnight at room temperature. Fibers were sputtered with a thin layer of carbon and examined with a Zeiss Evo LS10 Scanning Electron Microscope. For comparison with a normal bone trabecula, a sample of clean and dried human trabecular bone was prepared and analyzed as above.

Transmission electron microscopy (TEM)

After a 21-day period of incubation with J774.2 cells, the fibers were prepared for TEM. Cell fixation on the fibers was done with glutaraldhehyde 2.5% in phosphate buffer as described above and fibers were embedded in Lowicryl® K4M. Ultrathin sections were obtained with a diamond knife on a Leica Ultracut S and transferred onto copper grids (mesh 200) coated with a film of Formvar. Grids were then contrasted with uranyl acetate and lead citrate. Examinations were done with a JEOL JEM 1400 electron microscope (JEOL, Paris, France).

Animals and surgical procedure

Four New Zealand rabbits (15-16 week old and approximately weighting 3.5-3.75 kg) were used in the present study. They were purchased from Charles River (L'Arbresle, France) and acclimated during 8 days to the local stabling conditions. They received synthetic food and water *ad libitum*. The care and stabling of the laboratory animals were done at ONIRIS, the veterinary school of Nantes and all procedures were approved by the local ethical committee (decision #49028). They were operated by a trained surgeon (E.A.) in compliance with French law on animal experimentation. Animals were operated under general anesthesia with medetomidine (Domitor®, Pfizer, Paris France) and ketamine (Imalgène 1000®, Merial SAS, Villeurbanne France). The distal femoral

extremity of each femur was processed simultaneously during the same surgical time. After skin incision, and lateral arthrotomy access via the knee joint, a cylindrical defect (4 mm in diameter and 6 mm long) was created on the outer femoral condyle with an electric drill operating at low speed. After flushing with saline to eliminate the bone and marrow debris, the syringe containing the fiber scaffold (which was prior sterilized under UV radiations 260 nm wavelength for 24 hours), was placed on the hole performed on the cortice and the fibers were gently pushed inside the bone defect. Incision was then closed on 3 layers with resorbable sutures (Vicryl™, Ethicon, Issy les Moulineaux, France). The animals were left to heal for 60 days. Euthanasia was done by injecting an intracardiac overdose of sodium penthobarbital (Dolethal®, Vetoquinol France). The lower femoral extremities were immediately dissected from each animal and fixed in an alcohol-formalin based fixative (BB's).

X-ray microcomputed tomography (microCT)

A Skyscan 1172 X-ray computed microtomograph (Bruker microCT, Kontich, Belgium) was used with the cone beam mode to image the fiber scaffold and the implanted bones. Scaffolds of fibers compacted in a syringe (ready to use for animal study) [29] were analyzed at a magnification of ×15.36 (a pixel corresponding to 20 µm) at 80kV, 100 µA with no aluminum filter and a 0.25° rotation angle was applied at each step. Bones were scanned when in the fixative by using a 1mm aluminum filter; the pixel size was fixed at 18 µm and a 0.20° rotation angle was applied at each step. Briefly, serial section were acquired and stored in the *.bmp format. After interactive segmentation, the 3D models were constructed from the stack with a surface-rendering program (Ant, release 2.5.0.2,

Skyscan). Porosity, mean diameter of the pores and inter connectivity of the pores were determined on the 3D models using the CtAn software (Bruker) as previously described [29,30].

Histological analysis

Femurs were embedded undecalcified in poly(methyl methacrylate). They were cut-dry (7 µm in thickness) on a heavy-duty microtome equipped with tungsten carbide knives (Leica Polycut S - Rueil Malmaison, France). Sections were stained with a modified Goldner trichrome and analyzed on an Olympus BX 51 microscope equipped by bright field and polarization illumination.

Results

Cell viability assay

The cytotoxicity results obtained on SaOs2 and J774.2 cells revealed that polymer fibers made by the wet-spinning method have a low cytotoxic effect (Fig. 2). The percentage of necrotic cells was not significantly different than control after 24 h of incubation time. After 48 h of incubation the number of dead cells was higher than the control ($p < 0.05$), but the percentage remains close to 6%, which can be considered as an acceptable value. Accordingly it can be concluded that *in vitro* results showed a low cytotoxic response for two cell lines (osteoblasts and macrophages) after 24 h of incubation.

Scanning electron microscopy (SEM)

The diameter of fibers obtained through this method was in the range of 100–200 µm, as measured on SEM micrographs, using the ImageJ software (NIH - https://imagej.nih.gov/ij/). The fibers appeared to have a rough surface (Fig. 3A and 5A),

with a roughness similar to that of a bone trabecula containing calcified collagen microfibers (Fig. 3B).

SEM examination of incubated PHBV fibers with the two cell lines showed that they were able to adhere onto the scaffolds without any visible signs of suffering (blebbing, apoptosis or necrosis). SaOs2 cells were anchored onto the fibers after 24 h of incubation and at 7 days post-seeding they almost reached confluence on the surface of the polymer fibers (Fig. 3C). At this time point, the osteoblasts morphology was specific for well adherent cells, covering a high area of the biomaterial surfaceand establishing numerous filopodia; orientation of the cells along the longitudinal axis of each fiber was evidenced (Fig. 3C). Similar results were noticed in case of cells of the J774.2 line, but signs of polymer erosion could be evidenced at the surface of the fibers with minute erosion pits (Fig. 3D).

Transmission electron microscopy (TEM)

The polymer itself appeared as a regular scaffold with internal microporosity of about 0.5-1 µm. Examination of ultrathin sections of PHBV fibers incubated during 21 days showed that J774.2 cells have adhered and were affixed onto the polymer surface. It also showed that the J774.2 macrophages have engulfed small amounts of the polymer in their cytoplasm (Fig. 4). The identification of the polymer in the cell endosomes is an obvious sign of erosion.

MicroCT of fibers and grafted areas

The PHBV fibers could be easily observed (Fig. 5A) and frequently appeared to have a central cavity. When the fibers were packed together, they formed a 3D scaffold easily manageable for implantation (Fig. 5B). The porosity of the scaffold was found to be 79.7

± 3.3% with large pores (1830 ± 585 m in diameter) and high interconnectivity (inter connectivity index 192.73 ± 18.4).

On histological sections of bone grafted areas, the mineralized trabeculae of the bone around the hole appeared in green and the uncalcified periosteum in red (Figure 5C). The PHBV fibers were not stained by the Goldner trichrome but were birefringent under polarized microscopy (Figure 5D). No bone trabecular was observed between the PHBV fibers and no sign of erosion of the fibers was seen since they maintained their ovoid shape. The edges of the hole were not remodeled. The only finding was the development of a non-inflammatory vascularized fibrosis extending from the edges of the hole to the center of the grafted area, these areas of fibrosis appeared in red since they were not mineralized. No accumulation of inflammatory cells and no giant cell were observed around the fibers.

MicroCT examination of all animals revealed that no bone apposition has taken place between the fibers (Fig. 5E and 5F). The drilled holes were not filled by new bone trabeculae and only a minimal remodeling occurred at the edges of the hole.

Discussion

Due to the diversity of PHAs, their different properties (physicochemical and physical-mechanical properties), as well as their availability (being produced by microorganisms) make them a appealing for various biomedical applications. In our study, we tested fibers obtained using a simple method (wet-spinning method) from a commercial copolymer PHBV (purchased from GoodFellow SARL - France) as a bone grafting material, *in vitro* and *in vivo*.

PHBV (2% valerate) 20% (w/v) chloroform solution was found to be the optimal concentration for wet spinning of continuous fibers. Lower concentrations resulted in the absence of fiber formation of the extruded polymer solution. To be considered as tissue-engineering biomaterials, PHBV fibers must exhibit a good biocompatibility, with non-toxic degradation products [31]. The fibers obtained in the present study were relatively thick in the range of 100-200µm. Several authors have tried to prepared thinner fibers by electrospinning and have obtained nanofibrers of 1-3.5µm thick fibers [32,33]. However, it has been shown that thick fibers are preferable because osteoblast are more able to spread and anchored on them than on nanofibers [34].

The surfaces and interfaces of a biomaterial are areas where some of material properties can be different from the entire material due to their contact with biological fluids which can modify their properties [35]. Fibers obtained by the wet spinning method appeared to be a very convenient form to test the cytocompatibility of the material *in vitro*. Their diameters offer a large surface to cells to adhere, similar to the surface they encounter when apposed onto a bone trabecula [36]. Such a roughness is known to stimulate the adherence of cells, particularly osteoblasts [29,37]. Although each PHBV fiber is a separate entity, they can be combined by compaction to form an isotropic or anisotropic network which is highly porous through the generation of inter-fibrilar pores. Such scaffolds prepared by compacting the fibers were recently studied by different methods known to characterize porosity, including Euclidean and Fractal descriptors (succolarity and lacunarity) [29]. These fibrous scaffolds have a high and interconnected porosity that could favor cell and vascular sprouts invasion.

Cytocompatibility was appreciated by using *in vitro* experiments. For bone biomaterials, cytocompatibility can be better assessed using two types of cells that will come in direct

contact with the polymer after implantation: bone forming and bone resorbing cells, thereby enabling the study of formation and resorbability, respectively [38].

A biomaterial is considered to be a good candidate for bone grafting if it presents the following characteristics [39,40]: i) it should be osteconductive, i.e. the material must provide a scaffold to support new bone formation. Its surface must allow cells to attach, spread, proliferate, and deposit packets of bone matrix and finally, it should be resorbed after having been replace by new bone. ii) it should be osteogenic by having the capacity to stimulate bone healing. iii) it should be bioactive, i.e. it should allow the attachment and differentiation of osteogenic cells on its surface. iv) it should be osteoinductive, this implies the possibility for the biomaterial to act on mesenchymal cells by making them osteogenic and capable to appose new bone, even in a non-bony tissue such as muscle. This condition is in fact exceptionnaly observed. v) it should be mechanically resistant enough to be grafted in a weighting area (when used in orthopedic surgery).

A classification was previously established to describe the degradation modes of biomaterials and is particularly suitable for polymers [41]. A material is referred as biodegradable if it is degraded by macromolecular damage. It is not eliminated from the organism and debris can migrate far from the prosthesis (the best example is polyethylene or alumina wear debris coming from the hip/knee prostheses) [42-45]. In an *in vitro* study, we could show that the synthetic polymer poly(2-hydroxy ethylmethacrylate) could be degraded when placed in direct contact with the J774.2 cells [46]. A material is bioresorbable if it is degraded in bulk and entirely resorbed *in vivo*. It is eliminated from the organism as fragments of molecular low mass. Bioerosion concerns materials that are degraded in surface and then resorbed *in vivo*. A material is absorbable if it can dissolve in the organism without modification of molecular weight of its composing molecules.

In the present study, we found that the PHA polymer was erodible by macrophage-like cells *in vitro.* So, the PHA polymer appears to be bioactive and osteoconductive *in vitro* (i.e. cytocompatible). However, in a midterm implantation in the rabbit, no sign of degradation could be observed *in vivo* and no bone was apposed at the surface of the fibers. Furthermore, the fibers were encapsulated in a thin fibrosis. Although these fibers exhibited a good cytocompatibility *in vitro*, the material can only be classified as biotolerated [1]. A very similar finding was observed with electrospun microfibers of polystyrene, a synthetic non-resorbable biomaterial, which appeared highly cytocompatible but only biotolerated in the rabbit [47]. Other authors have proposed PHA scaffolds as bone substitutes, however, if their *in vitro* experiments confirmed cytocompatibility *in vitro*, the *in vivo* studies have not been evaluated [48].

Conclusion

PHBV fibers obtained by a wet spinning method appeared to be degradable by the J774.2 macrophage cell line *in vitro*. They were also colonisable by SaOs2 osteoblast- like cell which can spread and develop onto their surface. However, the material is soft and not mechanical resistant enough, so it did not favor bone apposition *in vivo*. Other applications of PHBV fibers are currently under study in our laboratory as a coating material or in other types of tissues.

Acknowledgments

The work has been funded by the Sectoral Operational Programme Human Resources Development 2007-2013 of the Romanian Ministry of Labour, Family and Social Protection through the Financial Agreement POSDRU/88/1.5/S/61178. This work was also made possible by grants from the ANR LabCom “NextBone” research program.

Conflict of Interest

No conflicts of interest, financial or otherwise, are declared by the authors.

Legends of Figures:

Figure 1 Chemical structure of PHA homopolymers.

Figure 2 Cytotoxic test of PHBV fibers in presence of SaOs2 osteoblast-like cell line after 24 and 48 hours of incubation and J774.2 macrophage-like cell line after 24 h.

Figure 3 A. Scanning electron microscopy aspect of the surface of a PHBV fiber obtained by the wet spinning method. Note the roughness of the surface which mimics the aspect of collagen fibers. B. SEM of a normal trabecular surface showing the roughness due to the presence of fully mineralized collagen fibers. C. SaOs2 cell spread on the surface of PHBV fibers after 7 days in culture. The cells are elongated along the groves of the polymer surface (BSE mode). D. J774.2 cells at the surface of a PHBV fiber. These round macrophage-like cells have eroded the material by creating small pits at the surface (arrow) after 21 days.

Figure 4 Transmission electron microscopic image of a J774.2 cell in direct contact with the PHBV polymer at 21 days post-culture. The cell contains numerous vacuoles (arrows). The polymer has a reticulated aspect and appears as a white network at the bottom of the image (*Polym*). Cartridge: enlargement of an endocytic resorption vacuole (arrow) containing a fragment of the polymer.

Figure 5 A. Fluorescence microscopy of the PHBV fibers showing surface roughness and irregularities. B. MicroCT analysis of the PHBV fibers packed together before

implantation. C. Light microscopy of the implanted area in a rabbit femoral condyle during 60 days. The PHBV fibers are packed together but do not exhibit any sign of degradation. The remaining mineralized bone around the hole appears in green (*mb*); it is covered by the unmineralized fibers of the periosteum (*p*). Note the minimal remodeling on the edge of the hole, particularly in the edge near the periosteum. The PHBV fibers are unstained and appear as white profiles (*f*). Thin layer of collagen fibrosis (in red) (*fib*) encapsulate all fibers (Goldner trichrome staining). D. same microscopic field analyzed under polarization microscopy, the fibers are clearly birefringent. E. MicroCT, 2D section of a rabbit femoral condyle implanted with PHBV fibers during 60 days. No bone is present inside the hole whose architecture is not reconstructed. A minimal remodeling has occurred at the edges of the hole. F. 3D reconstruction of the same bone exposing the internal part of the hole. Note the partial reconstruction of the edges of the drilled hole.

References

[1] Athanasiou KA, Agrawal CM, Barber FA, Burkhart SS. Orthopaedic applications for PLA-PGA biodegradable polymers. Arthroscopy 14;**1998**:726-37.

[2] Suh H, Hwang YS, Lee JE, Han CD, Park JC. Behavior of osteoblasts on a type I atelocollagen grafted ozone oxidized poly L-lactic acid membrane. Biomaterials 22;**2001**:219-30.

[3] Müller H-M, Seebach D. Poly(hydroxyalkanoates): A Fifth Class of Physiologically Important Organic Biopolymers? Angewandte Chem 32;**1993**:477-502.

[4] Pompe T, Keller K, Mothes G, Nitschke M, Teese M, Zimmermann R et al. Surface modification of poly(hydroxybutyrate) films to control cell–matrix adhesion. Biomaterials 28;**2007**:28-37.

[5] Freier T, Kunze C, Nischan C, Kramer S, Sternberg K, Saß M et al. In vitro and in vivo degradation studies for development of a biodegradable patch based on poly(3-hydroxybutyrate). Biomaterials 23;**2002**:2649-57.

[6] Kim JS, Lee BH, Kim BS. Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) by Ralstonia eutropha. Biochem Engin J 23;**2005**:169-74.

[7] Degeratu CN, Zaharia C, Tudora MR, Tucureanu C, Hubca G, Salageanu A et al. Influence of Porosity Upon Cells Adhesion on Polyhydroxyalkanoates Films. Chem Bull Politehnica Univ Timisoara 55;**2010**:189-92.

[8] Lemoigne M. Produits de dehydration et de polymerisation de l'acide ß-oxobutyrique. Bull Soc Chim Biol 8;**1926**:770-82.

[9] Freier T. Biopolyesters in Tissue Engineering Applications. In: Werner C, editor. Polymers for Regenerative Medicine, Vol. 203: Springer Berlin Heidelberg; 2006. p. 1-61.

[10] Williams SF, Martin DP. Applications of Polyhydroxyalkanoates (PHA) in Medicine and Pharmacy. Biopolymers Online: Wiley-VCH Verlag GmbH & Co. KGaA; 2005.

[11] Madison LL, Huisman GW. Metabolic Engineering of Poly(3-Hydroxyalkanoates): From DNA to Plastic. Microbiol Mol Biol Rev 63;**1999**:21-53.

[12] Steinbüchel A. Biopolymers. Weinheim: Wiley-VCH;2003.

[13] Peschel G, Dahse H-M, Konrad A, Wieland GD, Mueller P-J, Martin DP et al. Growth of keratinocytes on porous films of poly(3-hydroxybutyrate) and poly(4-hydroxybutyrate) blended with hyaluronic acid and chitosan. J Biomed Mater Res - Part A 85A;**2008**:1072-81.

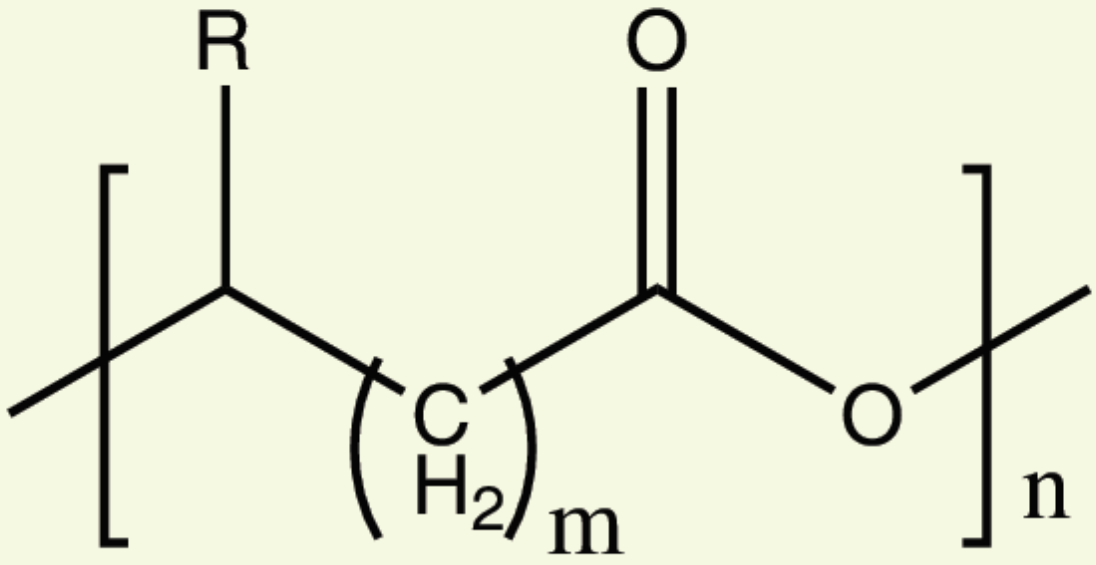
[14] Sun M, Zhou P, Pan L-F, Liu S, Yang H-X. Enhanced cell affinity of the silk fibroin- modified PHBHHx material. Journal of Material Science - Materials in Medicine 20;**2009**:1743-51.

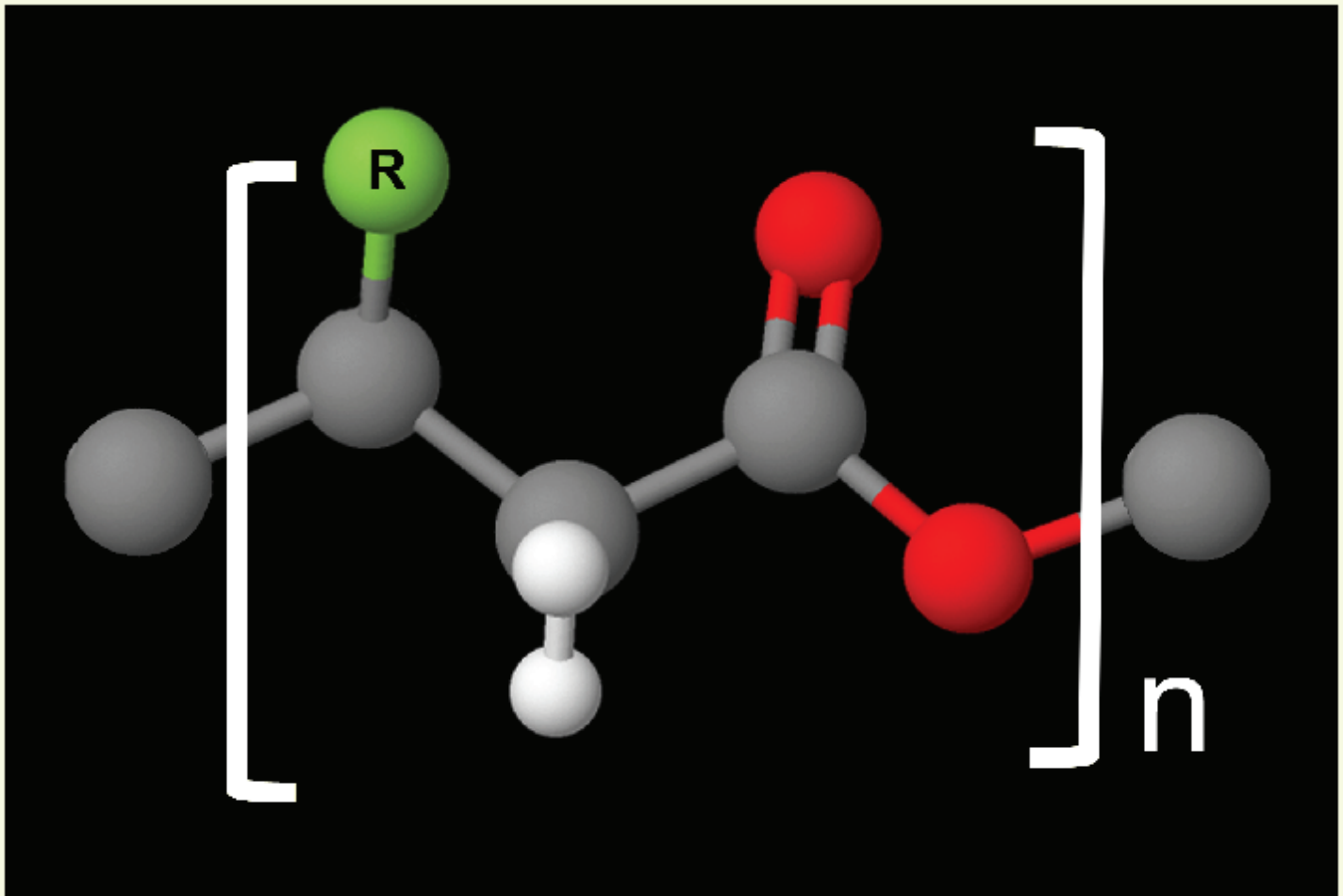
[15] Hu SG, Jou CH, Yang MC. Protein adsorption, fibroblast activity and antibacterial properties of poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) grafted with chitosan and chitooligosaccharide after immobilized with hyaluronic acid. Biomaterials 24;**2003**:2685-93.

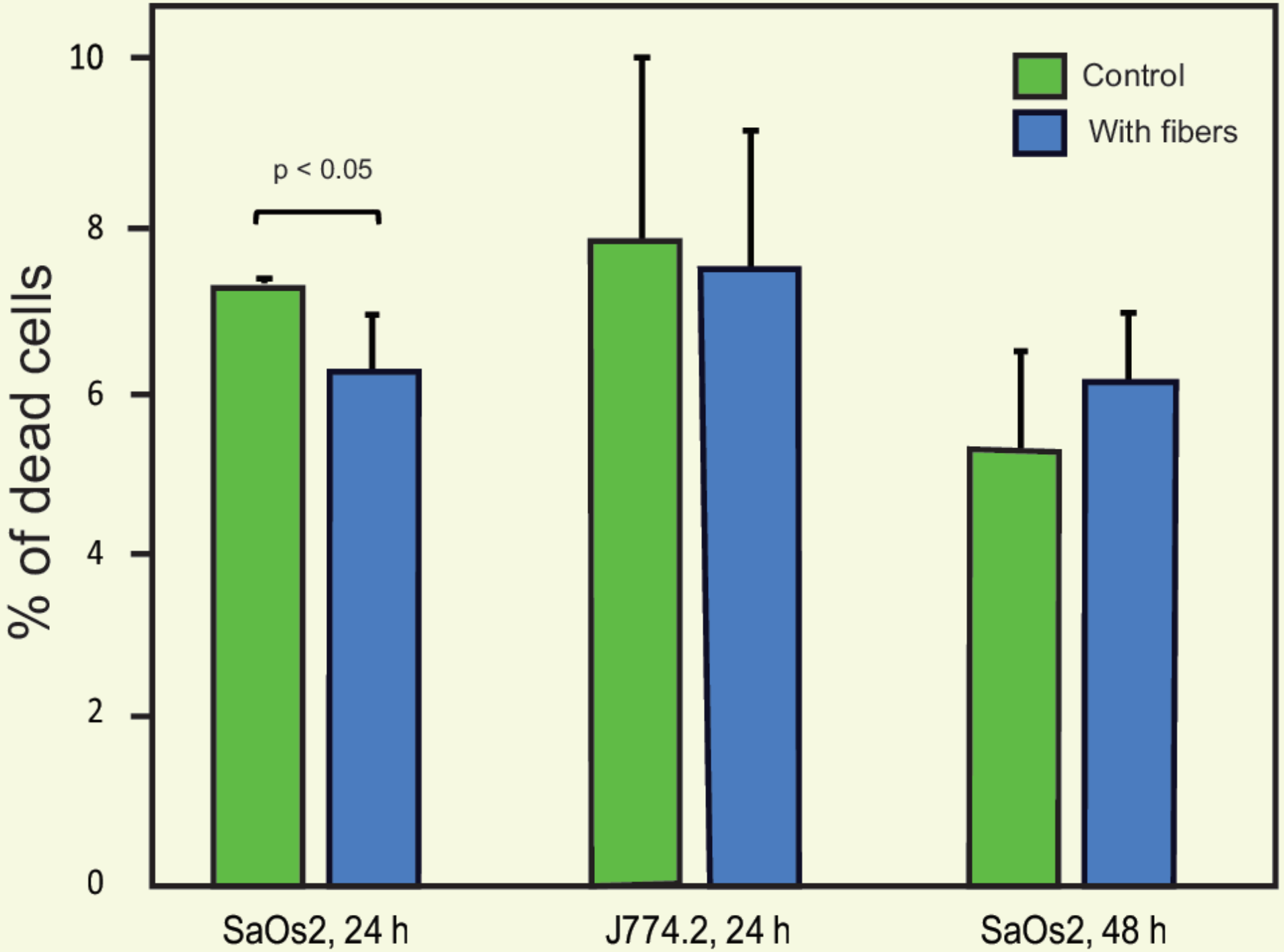
[16] Hu Y-J, Wei X, Zhao W, Liu Y-S, Chen G-Q. Biocompatibility of poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate) with bone marrow mesenchymal stem cells. Acta Biomater 5;**2009**:1115-25.
[17] Rezwan K, Chen QZ, Blaker JJ, Boccaccini AR. Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. Biomaterials 27;**2006**:3413-31.
[18] Tong HW, Wang M, Lu WW. Electrospun Poly(Hydroxybutyrate-co-Hydroxyvalerate) Fibrous Membranes Consisting of Parallel-Aligned Fibers or Cross-Aligned Fibers: Characterization and Biological Evaluation. J Biomater Sci Polym Ed 22;**2011**:2475-97.
[19] Meng W, Kim S-Y, Yuan J, Kim JC, Kwon OH, Kawazoe N et al. Electrospun PHBV/collagen composite nanofibrous scaffolds for tissue engineering. J Biomater Sci Polym Ed 18;**2007**:81-94.
[20] Cheng S, Wu Q, Yang F, Xu M, Leski M, Chen GQ. Influence of dl-β-Hydroxybutyric acid on cell proliferation and calcium influx. Biomacromolecules 6;**2004**:593-97.
[21] Kashiwaya Y, Takeshima T, Mori N, Nakashima K, Clarke K, Veech RL. D-beta-hydroxybutyrate protects neurons in models of Alzheimer's and Parkinson's disease. Proc Natl Acad Sci U S A 97;**2000**:5440-4.
[22] Tieu K, Perier C, Caspersen C, Teismann P, Wu DC, Yan SD et al. D-beta-hydroxybutyrate rescues mitochondrial respiration and mitigates features of Parkinson disease. J Clin Invest 112;**2003**:892-901.
[23] Zou Z, Sasaguri S, Rajesh KG, Suzuki R. dl-3-Hydroxybutyrate administration prevents myocardial damage after coronary occlusion in rat hearts. Am J Physiol Heart Circ Physiol 283;**2002**:H1968-74.
[24] Tasaki O, Hiraide A, Shiozaki T, Yamamura H, Ninomiya N, Sugimoto H. The dimer and trimer of 3-hydroxybutyrate oligomer as a precursor of ketone bodies for nutritional care. JPEN J Parenter Enteral Nutr 23;**1999**:321-5.
[25] Seal BL, Otero TC, Panitch A. Polymeric biomaterials for tissue and organ regeneration. Mater Sci Engin R 34;**2001**:147-230.
[26] Ratner BD, Bryant SJ. Biomaterials: Where we have been and where we are going. Annual Review of Biomedical Engineering 6;**2004**:41-75.
[27] Lee S-H, Shin H. Matrices and scaffolds for delivery of bioactive molecules in bone and cartilage tissue engineering. Adv Drug Deliv Rev 59;**2007**:339-59.
[28] Filmon R, Chappard D, Baslé MF. Scanning and Transmission Electron Microscopy of Poly 2-Hydroxyethyl Methacrylate-Based Biomaterials. J Histotechnol 20;**1997**:343-46.
[29] N'Diaye M, Degeratu C, Bouler J-M, Chappard D. Biomaterial porosity determined by fractal dimensions, succolarity and lacunarity on microcomputed tomographic images. Materials Science and Engineering C 33;**2013**:2025-30.
[30] Filmon R, Retailleau-Gaborit N, Grizon F, Galloyer M, Cincu C, Baslé MF et al. Non-connected versus interconnected macroporosity in poly (2-hydroxyethyl methacrylate) polymers. An X-ray microtomographic and histomorphometric study. J Biomater Sci Polym Ed 13;**2002**:1105-17.

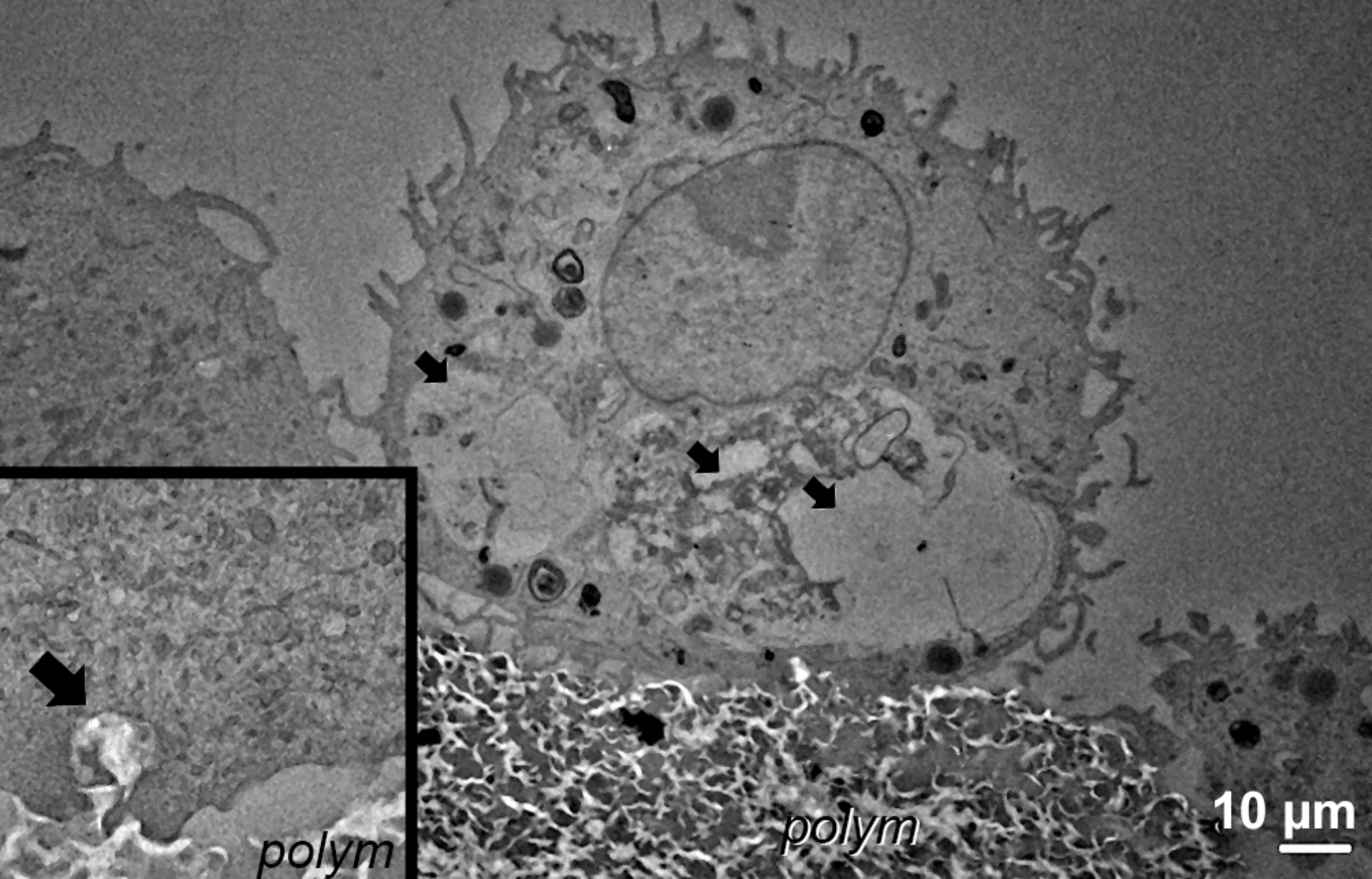
[31] Williams SF, Martin DP, Horowitz DM, Peoples OP. PHA applications: addressing the price performance issue: I. Tissue engineering. Int J Biol Macromol 25;**1999**:111-21.
[32] Volova T, Goncharov D, Sukovatyi A, Shabanov A, Nikolaeva E, Shishatskaya E. Electrospinning of polyhydroxyalkanoate fibrous scaffolds: effects on electrospinning parameters on structure and properties. J Biomater Sci Polym Ed 25;**2014**:370-93.
[33] Ying TH, Ishii D, Mahara A, Murakami S, Yamaoka T, Sudesh K et al. Scaffolds from electrospun polyhydroxyalkanoate copolymers: fabrication, characterization, bioabsorption and tissue response. Biomaterials 29;**2008**:1307-17.
[34] Terranova L, Mallet R, Perrot R, Chappard D. Polystyrene scaffolds based on microfibers as a bone substitute; development and in vitro study. Acta Biomater 29;**2016**:380-88.
[35] Cornet A, Deville JP. Physique et ingénierie des surfaces: ed. Sciences E;1998.
[36] Chappard D, Baslé MF, Legrand E, Audran M. Trabecular bone microarchitecture: A review. Morphologie 92;**2008**:162-70.
[37] Degasne I, Baslé MF, Demais V, Huré G, Lesourd M, Grolleau B et al. Effects of roughness, fibronectin and vitronectin on attachment, spreading, and proliferation of human osteoblast-like cells (Saos-2) on titanium surfaces. Calcif Tissue Int 64;**1999**:499-507.
[38] Beuvelot J, Pascaretti-Grizon F, Filmon R, Moreau MF, Basle MF, Chappard D. In vitro assessment of osteoblast and macrophage mobility in presence of beta-TCP particles by videomicroscopy. J Biomed Mater Res - Part A 96;**2011**:108-15.
[39] Bauer TW, Muschler GF. Bone graft materials: an overview of the basic science. Clinical Orthopaedics and Related Research® 371;**2000**:10-27.
[40] Blokhuis T, Arts JC. Bioactive and osteoinductive bone graft substitutes: definitions, facts and myths. Injury 42;**2011**:S26-S29.
[41] Vert M, Feijen J, Albertson A, Scott G, Chiellini E. Biodegradable polymers and plastics.: Cambridge, UK: Royal Soc. Chem.;1992.
[42] Libouban H, Massin P, Gaudin C, Mercier P, Baslé MF, Chappard D. Migration of wear debris of polyethylene depends on bone microarchitecture. J Biomed Mater Res B Appl Biomater 90;**2009**:730-7.
[43] Baslé MF, Bertrand G, Guyetant S, Chappard D, Lesourd M. Migration of metal and polyethylene particles from articular prostheses may generate lymphadenopathy with histiocytosis. J Biomed Mater Res 30;**1996**:157-63.
[44] Willert HG. Reactions of the articular capsule to wear products of artificial joint prostheses. J Biomed Mater Res 11;**1977**:157-64.
[45] Rony L, de Sainte Hermine P, Steiger V, Mallet R, Hubert L, Chappard D. Characterization of wear debris released from alumina-on-alumina hip prostheses: Analysis of retrieved femoral heads and peri-prosthetic tissues. Micron 104;**2018**:89-94.

[46] Mabilleau G, Moreau MF, Filmon R, Baslé MF, Chappard D. Biodegradability of poly (2-hydroxyethyl methacrylate) in the presence of the J774.2 macrophage cell line. Biomaterials 25;**2004**:5155-62.
[47] Terranova L, Dragusin DM, Mallet R, Vasile E, Stancu IC, Behets C et al. Repair of calvarial bone defects in mice using electrospun polystyrene scaffolds combined with beta-TCP or gold nanoparticles. Micron 93;**2017**:29-37.
[48] Lim J, You M, Li J, Li Z. Emerging bone tissue engineering via Polyhydroxyalkanoate (PHA)-based scaffolds. Mater Sci Engin C 79;**2017**:917-29.


R
O
C
H2
m
O
n


R
n

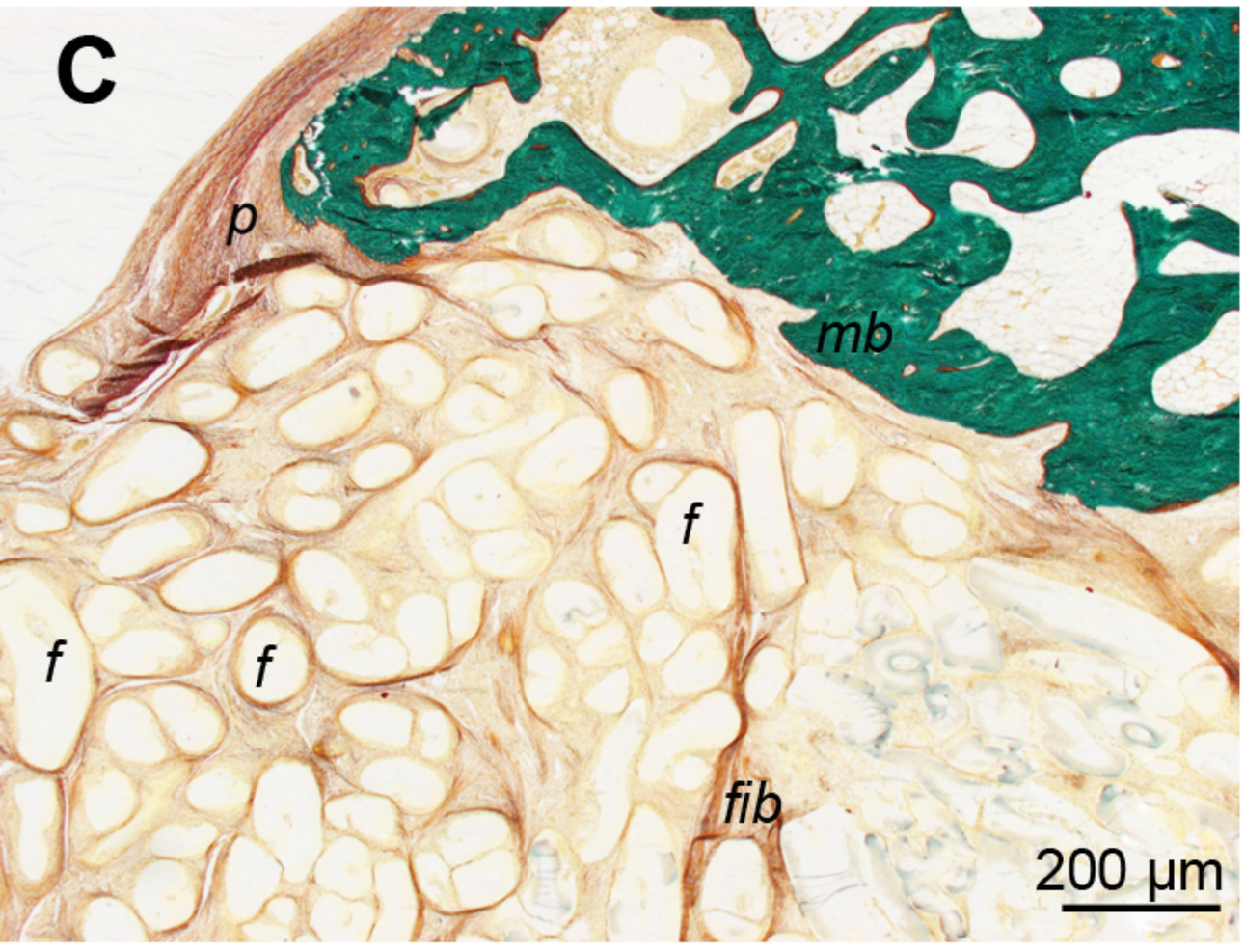

10
8
6
4
2
0
% of dead cells
p < 0.05
Control
With fibers
SaOs2, 24 h
J774.2, 24 h
SaOs2, 48 h


polym
polym
10 µm

A

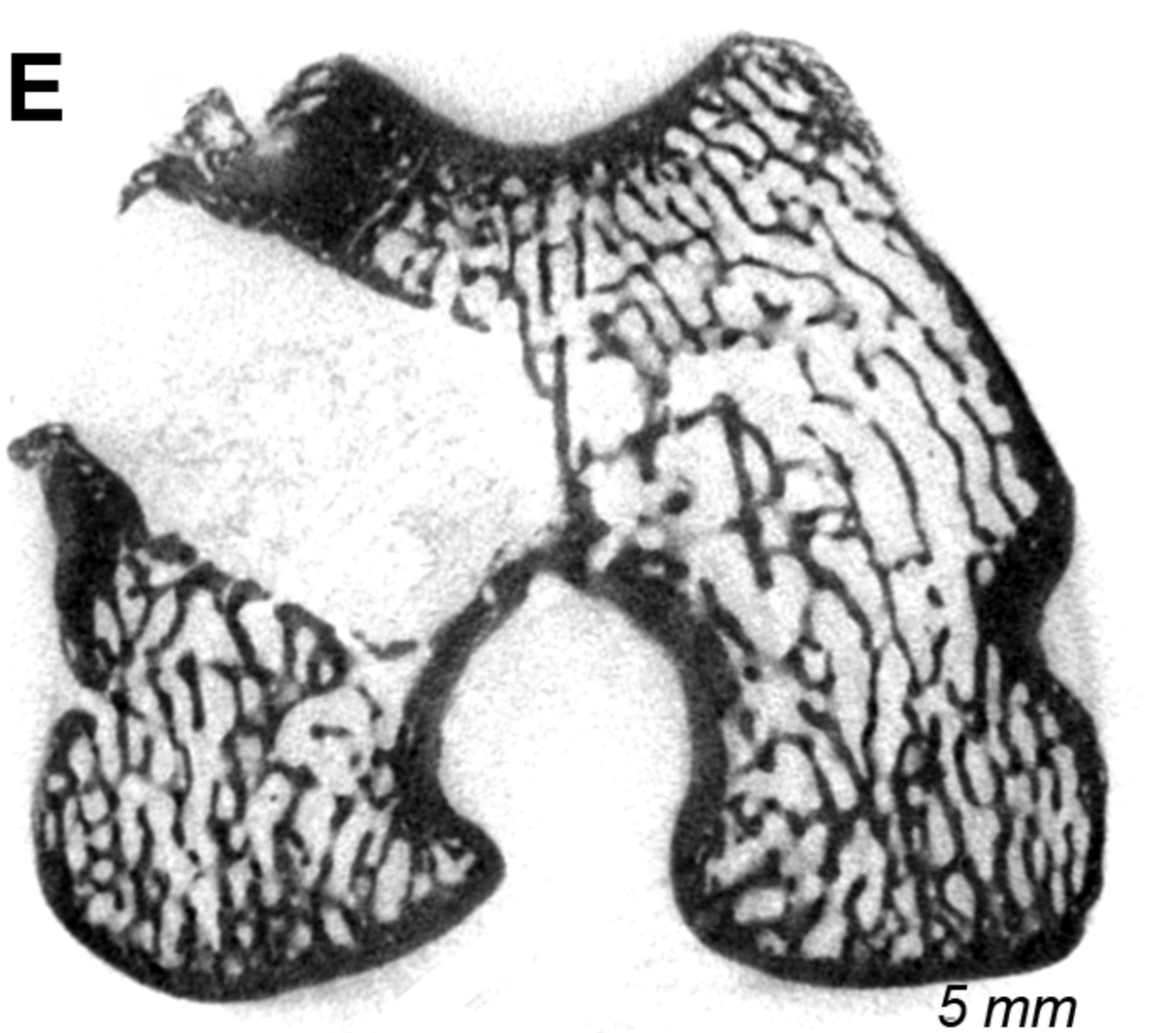
B

C



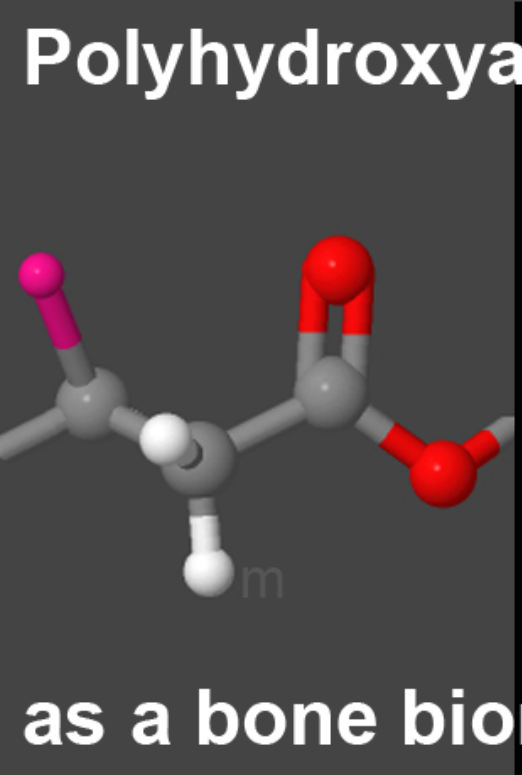
D

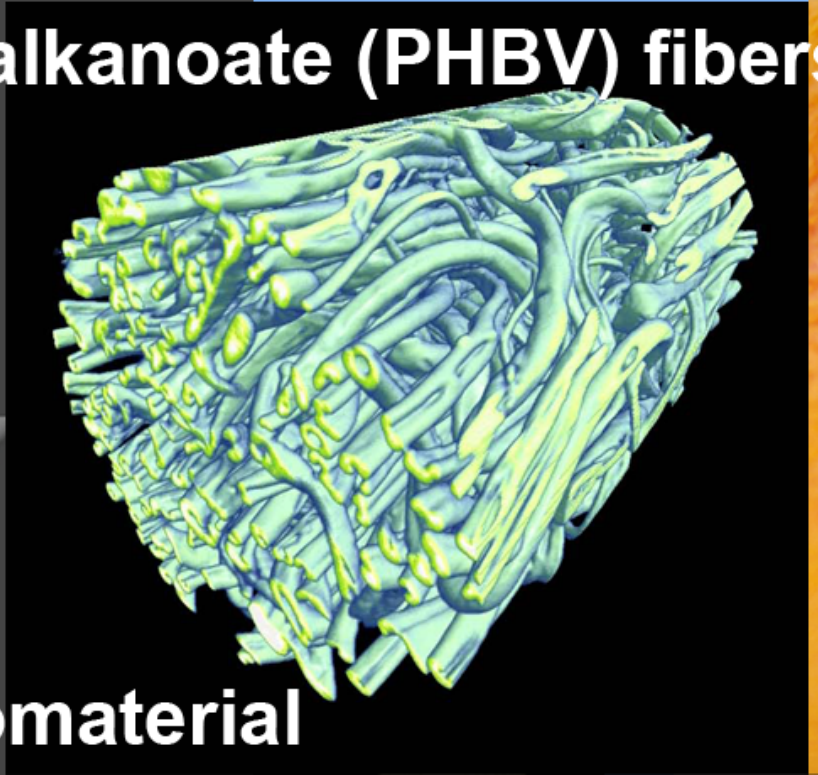
E



F

Polyhydroxyalkanoate (PHBV) fibers





as a bone biomaterial