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In vitro degradation and in vivo biocompatibility of chitosan–poly(butylene succinate) fiber mesh scaffolds

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Abstract

In tissue engineering, the evaluation of the host response to the biomaterial implantation must be assessed to determine the extent of the inflammatory reaction. We studied the degradation of poly(butylene succinate) and chitosan in vitro using lipase and lysozyme enzymes, respectively. The subcutaneous implantation of the scaffolds was performed to assess tissue response. The type of inflammatory cells present in the surrounding tissue, as well as within the scaffold, was determined histologically and by immunohistochemistry. In the presence of lipase or lysozyme, the water uptake of the scaffolds increased. Based on the weight loss data and scanning electron microscopy analysis, the lysozyme combined with lipase had a notable effect on the in vitro degradation of the scaffolds. The in vivo implantation showed a normal inflammatory response, with presence of neutrophils, in a first stage, and macrophages, lymphocytes, and giant cells in a later stage. Vascularization in the surrounding tissue and within the implant increased with time. Moreover, the collagen deposition increased with time inside the implant. In vivo, the scaffolds maintained the structural integrity. The degradation in vitro was faster and greater compared to that observed in vivo within the same time periods.

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Tissue engineering, chitosan, scaffold, biodegradation, biocompatibility, subcutaneous implantation

Introduction

Many biodegradable polymers have been proposed to produce scaffolds in tissue engineering. These structures sustain the extracellular matrix (ECM) production by cells and are expected to gradually degrade, allowing the surrounding tissue to replace the supporting function of the scaffold. Biodegradable polymers are used to provide temporary support for cell growth, degrading with time, in a controlled way into products, which can be eliminated by regular metabolic pathways in the body (biodegradation).[1] The biological performance of some biomaterials depends on their degradation behavior since this process influences cell performance and inflammatory response. Therefore, it is crucial to study the degradation properties of the scaffold for a long-term success of the tissue-engineered construct.[2]

Natural polymers have inherent characteristics that are interesting for specific tissue engineering applications.[3] Chitosan, the partially deacetylated product of chitin, has emerged as one of the preferred natural materials, as it is similar to glycosaminoglycans (GAGs), which are native components of ECM.[4] Additionally, the cationic nature of chitosan allows electrostatic interactions with GAGs and proteoglycans.[5] In our group, we have been working with chitosan-based scaffolds.[6–10] One of the most promising biomaterials is Chitosan–poly(butylene succinate (Ch-PBS)) processed into scaffolds by melt based technology, combining the biological properties of chitosan and the mechanical properties of aliphatic polyesters.[6,11,12] Chitosan has been proposed as biomaterial for biomedical applications mainly due to its biocompatibility.[13,14] Furthermore, it has been described as a potent wound healing accelerator,[15,16] as well as to modulate the immune system by activating macrophages[17] to produce cytokines and to inhibit infection.[18]

PBS is a biodegradable synthetic polymer with good processability when compared to poly(lactic acid) or poly(glycolic acid).[19] The degradation profile of PBS in the environment has been widely studied.[20,21] Succinic acid is the main degradation product, which is an intermediate of the tricarboxylic acid cycle, that ultimately degrades into carbon dioxide and water.[21] Some biomaterials are degraded by hydrolysis, being decomposed in contact with water or serum in a non regulated way.[22] Biodegradation of polymeric biomaterials requires cleavage of hydrolytically and/or enzymatically sensitive bonds in the polymer.[23] Degradation products should be nontoxic and free of immunogenicity. The resulting products should be small enough to dissolve in the body fluids and, after transportation via lymphatic system, the kidneys should be able to excrete them from the body.[24] The degradation behavior of polymers can be tested in vitro, to predict their in vivo behavior when implanted. The biomaterials are usually incubated in phosphate-buffered saline, with or without enzymes, at 37°C, under static or agitated conditions to simulate the in vivo conditions. It is common that both in vitro and in vivo degradation studies are performed in parallel with biocompatibility tests. This is due to the fact that the degradation of a biomaterial implant in a host is influenced by the presence and recruitment of inflammatory cells as well as by the production of inflammatory mediators.

In vivo, chitosan degrades mainly by the action of lysozyme.[25,26] The degradation kinetics of chitosan is inversely related to the degree of deacetylation[25,27] since this enzyme targets the acetylated residues of chitosan polymer.[26,28] Human lysozyme is found in several body fluids, including serum, tears, saliva, and other fluids, like those surrounding cartilage. During the inflammation process, neutrophils and macrophage cells release enzymes, such as lysozyme and reactive oxygen

species.[29] The degradation of aliphatic polyesters, such as PBS, is known to be catalyzed by lipases.[30] This enzyme is water-soluble, hydrolyzing ester bonds of triglycerides, phospholipids, and cholesteryl esters.[31] Human pre-duodenal (lingual and gastric) and extra-duodenal lipase is mainly derived from pancreatic cells, but the digestive tract, adipose tissue, lungs, and leukocytes also contain lipase.[32]

An important requisite for clinical application of a biomaterial is its biocompatibility.[33] The implantation of a biomaterial device sets off a cascade of events starting with acute inflammation that may lead to a chronic inflammatory response. Polymorphonucleated cells (PMNs), macrophages, and new blood vessels are present, and granulation tissue can be developed, with subsequent foreign body reaction (FBR) and fibrous capsule development.[34] The intensity and the duration of the inflammatory reaction are used to characterize the biocompatibility of a biomaterial.[35] In the case of biodegradable polymers, the intensity of these responses may be modulated by the biodegradation process that can cause changes in shape, size, surface roughness, porosity, and release of degradation products.[35] Generally, the local reaction of an implant is studied after a 3-month implantation period, as described in the ISO 10993-6:1994.[36] This time frame usually reflects a steady state, where the local acceptance/rejection can be evaluated.

In this work, we studied the in vitro biodegradation, as well as the in vivo biocompatibility of Ch-PBS fiber mesh scaffolds. The in vitro degradation studies were carried out using lysozyme and lipase, in concentrations similar to those present in human blood serum. In vivo studies were performed with the main aim of studying the biodegradation and the biocompatibility of these scaffolds, using a subcutaneous model in Wistar rats during 3 months of implantation.

Materials and methods

Scaffolds production

The processing methodology is described in our previous works. Briefly, chitosan (85% deacetylated) was melt blended with PBS by extrusion (50 wt%).[11] The extrude was ground into powder and processed into microfibers (≈500 μm) also by extrusion. The fibers (6.5 mm diameter and 1 mm width) were cut and hot compressed at 150°C for 10 min. The scaffolds were sterilized with ethylene oxide for subsequent tests.

In vitro degradation studies

Degradation studies were performed in triplicate by incubating the scaffolds in 10 mL of phosphate-buffered saline (pH 7.4; control), with lipase from *Aspergillus oryzae* (Fluka, USA) (110 U/L) and/or lysozyme from chicken egg white (Sigma, USA) (13 mg/L) at 37°C under agitation (60 r/min) for 1, 3, 6, and 12 weeks. The enzymatic concentrations used were similar to those found in human blood serum. At the end of each degradation period, the samples were removed and immediately weighed to determine the water uptake and dried for later calculation of the weight loss.

Water uptake and weight loss determination

Water uptake and weight loss measurements were performed by immersing the scaffolds in phosphate-buffered saline or enzymatic solutions at 37°C for up to 12 weeks. The swollen sample weights were measured after removing excess surface water by gently touching the surface with filter paper. Water uptake was determined from the data on swollen weight (after equilibration and

eventual degradation or partial solubilization, w_s) and the final dried weight (w_f) using equation (1). The average of three experiments was considered the water uptake value

$$\text{Water uptake (\%)} = \left[\frac{(w_s - w_f)}{w_f}\right] \times 100 \quad (1)$$

The weight loss was calculated from the initial dried weight w_i and final dried weight w_f using equation (2)

$$\text{Weight loss (\%)} = \left[\frac{(w_i - w_f)}{w_i}\right] \times 100 \quad (2)$$

All samples were weighed before incubation in phosphate-buffered saline or enzymatic solutions (initial weight). After 1, 3, 6, and 12 weeks, three samples of each condition were removed and immediately weighed for determination of water uptake (equation (1)), washed thoroughly with distilled water, and air dried for later calculation of weight loss (equation (2)).

Analysis of sample morphology by scanning electron microscopy

The morphology of the samples was analyzed after sputter coated with gold by scanning electron microscopy (SEM) (NanoSEM; FEI, USA).

In vivo biocompatibility assessment

One day before subcutaneous implantation surgery, the implants were immersed in phosphate-buffered saline under sterile conditions. In total, 12 adult male Wistar Han rats (41–44 days old at the beginning of the experiment), specified pathogen free, were purchased from Charles River Laboratories, France. The animals were kept 1 week in a quarantine room and transferred to a conventional maintenance room of the experimental unit of the animal facility, where they were housed for the full period of the experiment.

At 1, 3, 6, and 12 weeks, samples were retrieved for further analysis. Animals were anesthetized by an intraperitoneal injection of a solution of 75/0.5 mg/kg body weight ketamine/medetomidine (Imalgene®, Novavet, Portugal/Dorbenvet®, Pfizer, EUA). After confirming the depth of anesthesia by pedal reflex, the dorsum of the animals was shaved and then placed in ventral position. The incision site at the dorsal skin was disinfected with chlorhexidine where two medial longitudinal incisions were performed. Subcutaneous pockets were created, and four scaffolds were placed in each animal, away from the suture site (incision) to avoid inflammation of the wound. The incisions were closed with a 4.0 silk suture (LOOK; Harvard, USA), which was removed 10 days after surgery in the animals with longer implantation periods. The anesthesia was then reverted with a subcutaneous injection of 0.25 mg/kg Atipamezol (Antisedan®, Novavet, Portugal). When the animals became active, they were placed in their home cages and water and food were supplied ad libitum. Each animal received a subcutaneous injection of 1 mg/kg analgesic Butorphanol (Torbugesic®, Pfizer, EUA) administered immediately after surgery and 24 h later, to avoid postoperative pain. At each time point, animals were euthanized by intraperitoneal injection of sodium pentobarbital, at a lethal dose, and the respective implants were retrieved.

All procedures were conducted in accordance with European regulations for animal laboratory testing (European Union Directive 86/609/EEC).

Histological evaluation

Implant processing and hematoxylin and eosin staining

The implants were collected with the surrounding tissues, and histology was performed by fixing the implants in 10% neutral buffered formalin. The specimens were cut to obtain 3-µm-thick longitudinal and transversal sections to analyze the tissue integration within the scaffolds. Sections were stained with hematoxylin and eosin (H&E) to evaluate the cellular infiltration throughout the implants.

Masson's trichrome staining

Masson's trichrome stain was used to evaluate the amount and the distribution of mature collagen surrounding and within the implants. This stain was used to differentiate collagen from other fibers (e.g. smooth muscle and elastin fibers). Masson trichrome stains collagen green, nuclei black and cytoplasm red.

Immunohistochemistry

Immunostaining for alpha-smooth muscle actin (α-SMA, Abcam, United Kingdom) antibody was performed to assess the vascularization over time. Antigen retrieval was heat induced in a water bath at 96°C for 20 min, with incubation of the slides in citrate buffer (pH 6). The slides were washed with phosphate-buffered saline, and endogenous peroxidase was blocked with 0.6% hydrogen peroxide (H_2O_2) in methanol at room temperature (RT) for 30 min. R.T.U. VECTASTAIN® Universal Elite ABC Kit (VCPK-7200; Vector, United Kingdom) was used for antibody incubation, according to the instructions of the manufacturer. Sections were incubated overnight in a humidified atmosphere with primary antibody (ab5694; Abcam) at 4°C. After washing with phosphate-buffered saline, antibody detection was determined by using the DAB Peroxidase Substrate Kit (VCSK-4100; Vector). The slides were washed in water for 5 min and then counterstained with Harris' hematoxylin for nuclear contrast. All images were obtained using an Olympus BX61 Motorized System Microscope and an attached video camera (Olympus DP70).

Results and discussion

Weight loss and water uptake

The scaffolds based on Ch-PBS were prepared by melt spinning and fiber bonding.[11,12] The main aim of the in vitro degradation studies was to simulate physiological conditions using enzymes present in human serum, which are responsible for the degradation of Ch-PBS fiber mesh scaffolds. Degradation with lysozyme and/or lipase was performed under static and agitated conditions. No differences were observed by the pH in the different degradation solutions after each incubation time (data not shown).

The ability of a material to absorb water and its water permeability influence the absorption of body fluids and the transfer of cell nutrients and metabolites throughout the materials. The water uptake (Figure 1) by the scaffolds immersed in phosphate-buffered saline (control), in dynamic conditions without changing the solutions, was approximately 40% hydration. This value is not high compared with other chitosan scaffolds,[37–39] as the hydrophobic nature of PBS decreases the

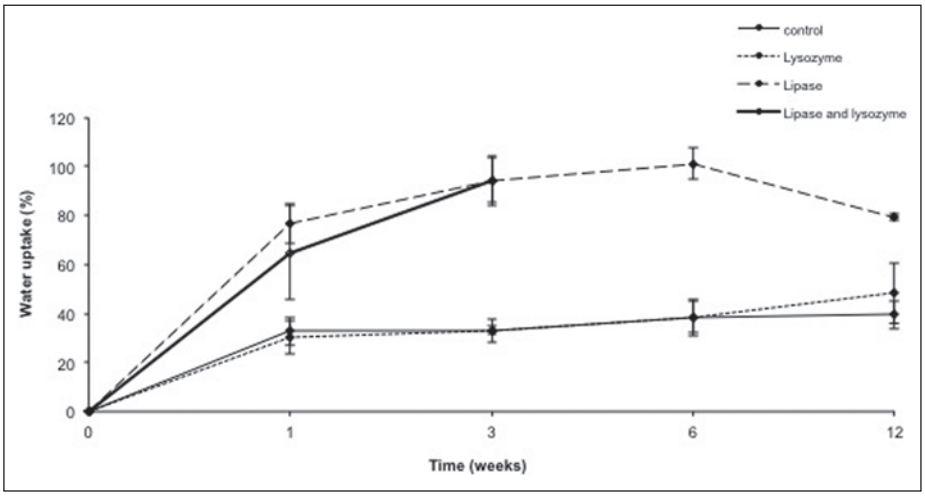

Figure 1. Water uptake of the scaffolds as a function of immersion time in phosphate-buffered saline with lysozyme (13°mg/L), lipase (110°U/L), and both lipase and lysozyme at pH 7.4, temperature of 37°C, under agitation conditions. Phosphate-buffered saline alone was used as a control.

absorption of water. Previous studies with compact disks of the same polymer blend presented a lower value of water uptake,[40] which is due to the sample morphology. A fiber mesh structure presents higher surface area than a compact structure. When only lysozyme was present, the degradation of the scaffolds was similar to the control (Figure 1). However, the water uptake of the materials increased remarkably in the presence of lipase or lipase with lysozyme (Figure 1), which could be due to degradation of the scaffolds in the presence of these enzymes.

When analyzing the weight loss profile of the scaffolds in the presence of lysozyme or lipase, only a few differences were observed (Figure 2). The degradation of chitosan is mediated by lysozyme.[25,26] The scaffolds immersed in phosphate-buffered saline supplemented with lysozyme presented the highest weight loss (5%) in the first week, as compared with the other conditions. The weight loss remained constant until the end of the experiment (Figure 2). The degradation of the scaffolds in the presence of lysozyme has similar values to that of the control at the end of the experiment. It is well documented in the literature that the degree of deacetylation is inversely related to the degradation rate.[27,28,41]

Lipase enzyme readily hydrolyses ester bonds in polyesters,[30,42] and therefore, the weight losses obtained were higher than those in the presence of lysozyme and increased with immersion time (Figure 2). The highest weight loss was observed in the presence of lipase and lysozyme together (Figure 2). These results are in agreement with previous studies using different enzymatic mixtures.[43,44] The immersion of the scaffolds up to 12 weeks did not cause any lose of structural integrity in the presence of either lysozyme (Figure 3(e) and (f)) or lipase (Figure 3(g) and (h)). However, lipase and lysozyme together induced loses of structural integrity of the scaffolds after 3 weeks. At the sixth week, all the scaffolds lost their structural integrity in the presence of both enzymes (Figure 3(i) and (j)).

Morphology of the scaffolds before and after in vitro degradation

The morphology of the scaffolds was analyzed by SEM, before and after degradation with the different solutions (Figure 3). It was possible to observe the surface of the fibers before degradation

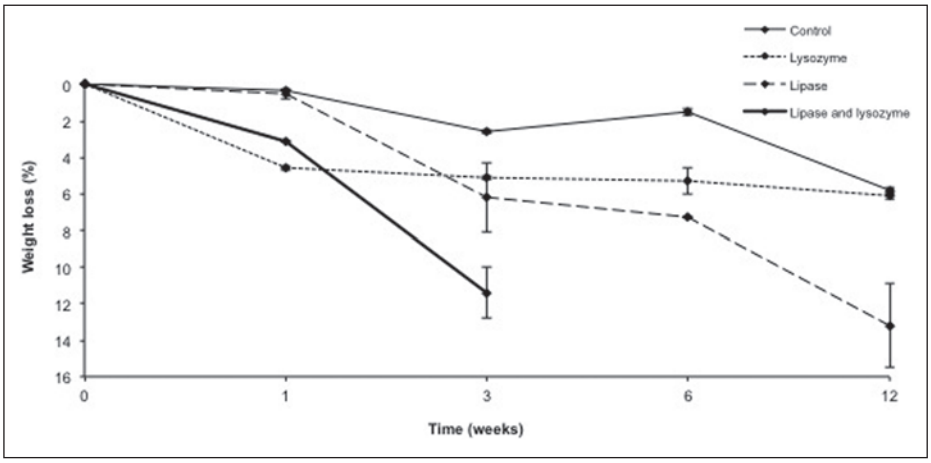

Figure 2. Weight loss of the scaffolds as a function of immersion time in phosphate-buffered saline with lysozyme (13°mg/L), lipase (110°U/L), and both lipase and lysozyme at pH 7.4, temperature of 37°C, under agitation conditions. Phosphate-buffered saline alone was used as a control.

(Figure 3(a) and (b)). After immersion in PBS solution, the surface of the fibers had fissures as a result of degradation (Figure 3(c) to (j)) and also a rougher surface (Figure 3(c) and (d)). The scaffolds incubated with lysozyme solution (Figure 3(e) and (f)) also presented fissures at the surface of the fiber, similarly to those incubated in phosphate-buffered saline. This may be explained by the fact that the chitosan used had a high degree of deacetylation causing lysozyme to have minimal effect on the polymer degradation.[28] However, for scaffolds incubated in lipase solution (Figure 3(g) and (h)), more fissures are visible at the surface of the fibers confirming that lipase was attacking the polyester phase. The combination of both lysozyme and lipase (Figure 3(i) and (j)), corroborated by the water uptake and weight loss resulted in larger fissures on the surface of the fibers.

In vivo biocompatibility assessment by histological analysis

No signs of infection were observed during the study or after surgery. Scaffolds were implanted subcutaneously, and explants were retrieved after 1, 3, 6, and 12 weeks. Local tissue integration, inflammatory response, and degradation behavior were assessed by histological stains (H&E and Masson's trichrome).

The implantation of a biomaterial may result in injury to tissues or organs.[34,45] The tissue response to injury depends on various factors, including the extent of the injury, blood–material interactions, extent or degree of cellular necrosis, provisional matrix formation, and the inflammatory response.[35] Materials currently used in clinical applications are considered nonimmunogenic, nontoxic, and chemically inert and elicit frequently acute and potential chronic inflammatory responses.[46]

Chitin and chitosan are known to accelerate wound healing and the attainment of a good healing surface. Based on histological findings, these substances stimulated the migration of polymorphonuclear and mononuclear cells and accelerated connective tissue regeneration and angiogenesis.[47] It is also known that chitosan has the ability to attract neutrophils and activate macrophages.[17,48]

Histological analysis of implanted scaffolds revealed that the porous morphology of the scaffolds was maintained after 7 days of implantation (Figure 4). The inflammatory infiltrates

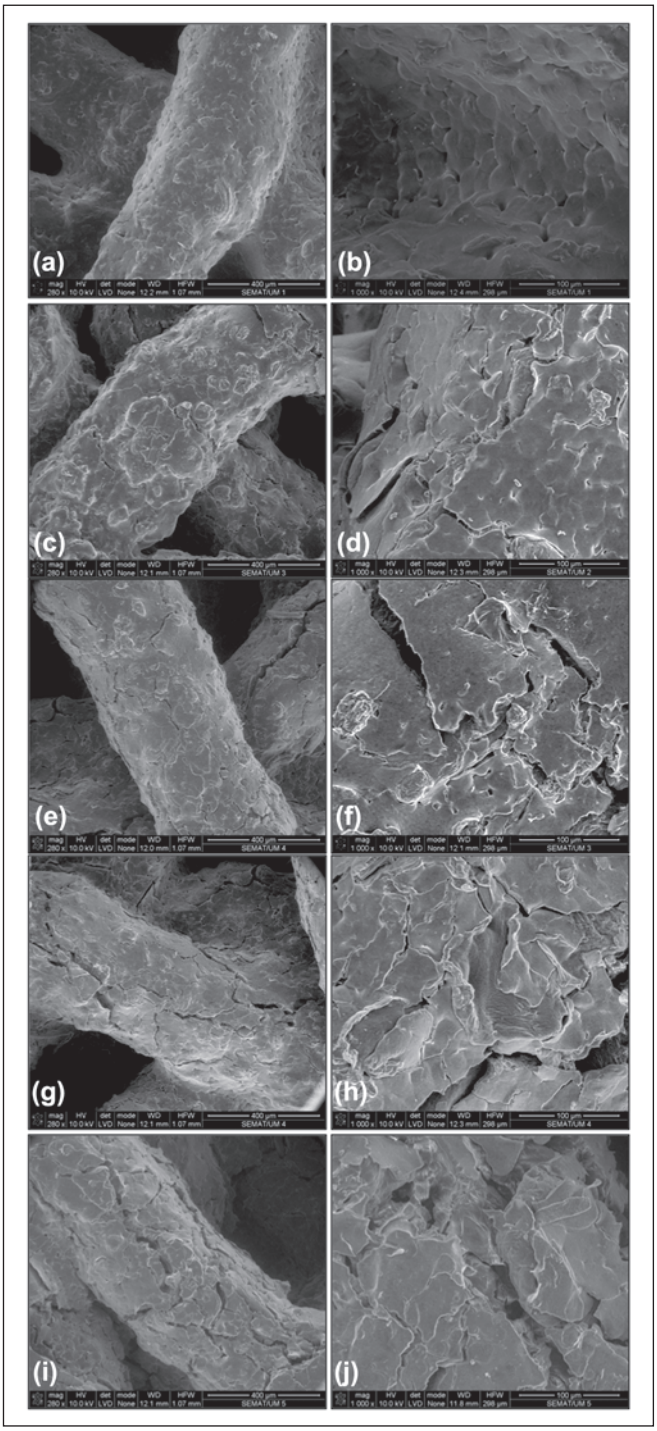

Figure 3. SEM micrographs of the morphology of chitosan–poly(butylene succinate) scaffolds (a and b) before degradation, (c and d) after 12 weeks in phosphate-buffered saline, (e and f) plus lysozyme, (g and h) lipase, and (i and j) both lysozyme and lipase.
SEM: scanning electron microscopy.

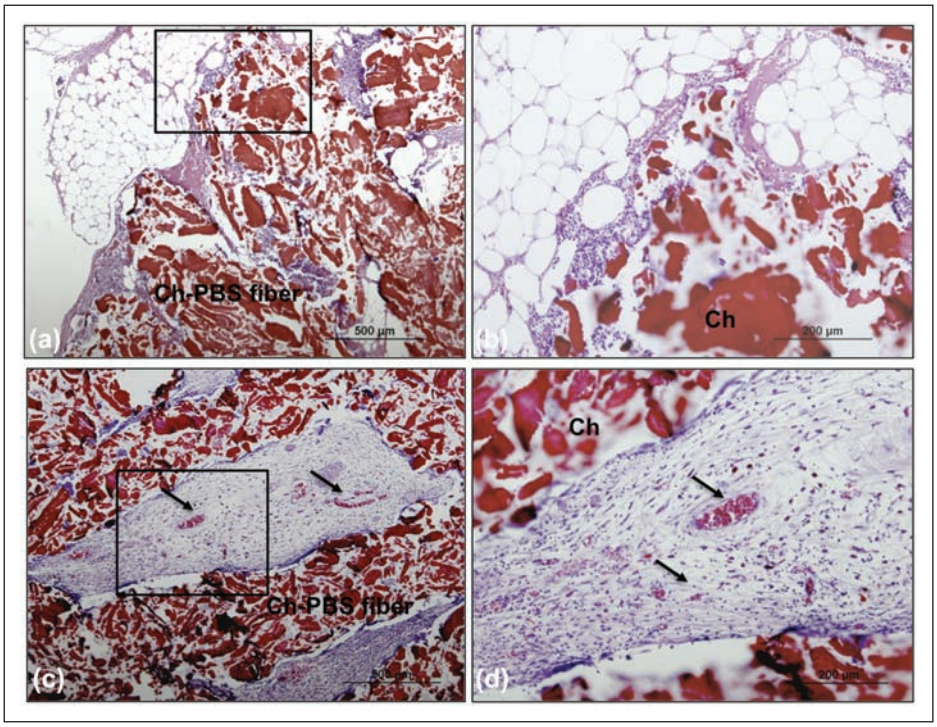

Figure 4. Representative H&E stained histological sections of tissues surrounding chitosan-based implants after (a and b) 1 week and (c and d) 3 weeks of subcutaneous implantation in Wistar rats; (b) and (d) represent the magnified sections of selected areas (squares) of (a) and (b), respectively. Black arrows point to blood vessels.
H&E: hematoxylin and eosin; Ch: chitosan; PBS: poly(butylene succinate).

were mainly constituted of neutrophils (Figure 4(b)). These cells were characterized by multilobulated nuclei, recruited from blood circulation, which reacted to the implanted Ch-PBS scaffolds. These cells are characteristic of the acute inflammatory response, which has short duration (hours to days), and are characterized by exudation of fluid and plasma proteins (edema) and the emigration of leukocytes, mainly neutrophils. The major role of these cells in acute inflammatory response is to phagocyte microorganisms and foreign materials. In the case of biomaterials, neutrophils are not able to phagocyte them because of the size disparity (Figure 4(b)).

At 3 weeks of implantation, the presence of neutrophils was almost gone. The presence of blood vessels within the scaffold structure was clear (Figure 4(c) and (d)). The SMA immunostaining was visible inside the fibers of the scaffold (Figure 5), which indicated that connective tissue was growing and vascularization was increasing throughout the scaffold.[8]

Concurrently, collagen was being deposited by fibroblasts (Figure 6). The appearance of blood vessels and fibrosis was an indication of a chronic inflammatory response. This type of response emerges when a persistent stimulus is present.

After 6 weeks of implantation (Figure 7(a) and (b)), the evolution of the acute inflammatory response into a chronic response was observed by the presence of granulomatous tissue and giant cells.

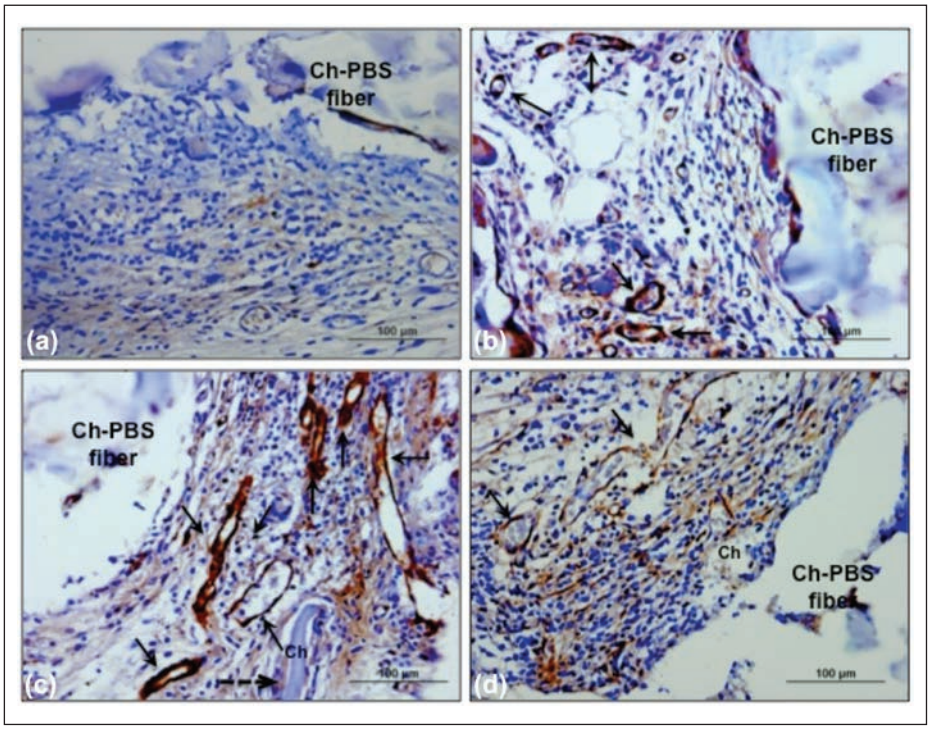

Figure 5. Representative α-SMA immunostained sections of tissues inside fibers of Ch-PBS mesh scaffolds after (b) 3 weeks, (c) 6 weeks, and (d) 12 weeks of implantation. (a) Negative control. Black arrows point to new blood vessels. Dashed arrow points to a phagocyted chitosan particle.
Ch: chitosan; PBS: poly(butylene succinate); SMA: smooth muscle actin.

Chronic inflammatory response is characterized by the presence of mononuclear cells, which includes macrophages, lymphocytes, and plasma cells.[49] Implantation of foreign materials elicits the foreign body reaction (FBR), composed of foreign body giant cells and granulation tissue development, constituted by macrophages, fibroblasts, and capillaries.[35] Biodegradable materials cause FBR which with time will become chronic until final degradation. In the case of nondegradable materials, the reaction continues until a capsule is formed around the implant, isolating it and FBR from the local tissue environment.[46] Foreign body giant cells are formed when material particles are too large to be phagocyted by macrophages and these cells fuse. In Figure 5(c), the phagocytosis of a chitosan particle by giant cells is observable (dashed arrow). A major organization of the tissue within the implant was observed with a marked presence of α-SMA (Figure 5(c)) and collagen deposition (Figure 6(c)).

At the end of the 12th week, organized tissue around and within the implanted scaffold was observed (Figure 7(c) and (d)) with collagen deposition (Figure 6(d)), adipocytes (Figure 7(c) and (d)), and a substantial increase in vascularization of the neo-formed tissue (Figure 6(d)). Moreover, it was visible that cells started to colonize the interior of the fibers (Figure 7(c) and (d)), which means that polymers were being degraded. Additionally, more blood vessels were present (Figure 6(c)), and some adipocytes (Figure 6(d)) in the surroundings of the implantation site were observed.

Up to 3 months, there were no signs of significant degradation of the implanted scaffolds (Figure 8). Histological findings only revealed the ingrowth of cells within each fiber of the scaffold

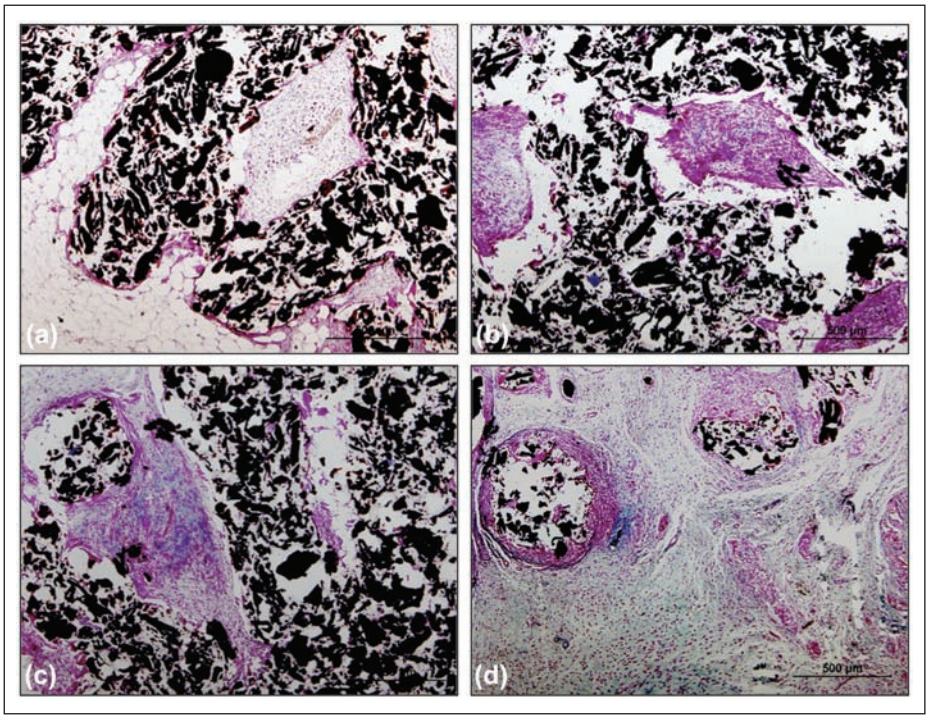

Figure 6. Masson's trichrome stained sections of tissues of chitosan–poly(butylene succinate) mesh scaffolds after (a) 1 week, (b) 3 weeks, (c) 6 weeks, and (d) 12 weeks of implantation. Green stain is collagen. The bar corresponds to 500 µm.

(Figure 7(c) and (d)). There was some evidence that giant cells attempted to phagocyte the particles of the scaffold (Figure 7(c)).

After 12 weeks of implantation, all scaffolds maintained their shape and structure (Figure 8), in contrast with the in vitro results, in which after 6 weeks of incubation in lipase and lysozyme, the scaffolds lost their structural integrity (Figure 2). Thus, it is clear that in vitro degradation was much faster than in vivo degradation. One fact that must be highlighted is that the magnitude of tissue response to a biodegradable material depends upon the site of implantation.[50] It was our aim to mimic the in vivo environment with enzymes that are present in the human body and are specific for the degradation of chitosan and the polyester. However, the in vivo results were not similar to those of in vitro, and this can be explained based on the activity of the enzymes. Another issue is the type of the enzymes used, which even at concentrations similar to the ones in human serum are not from human origin and their action might be slightly different. Furthermore, in vitro tests provide a much more hydrated environment than the host environment in vivo.

Conclusion

The degradation rate of the scaffolds must be tailored appropriately accordingly with the growth rate of the new tissue. The enzymatic mixture with lysozyme and lipase had a strong positive effect on the scaffolds degradation. The in vitro degradation studies were carried out using lysozyme and lipase, in concentrations similar to those present in human blood serum. After 12 weeks of in vivo

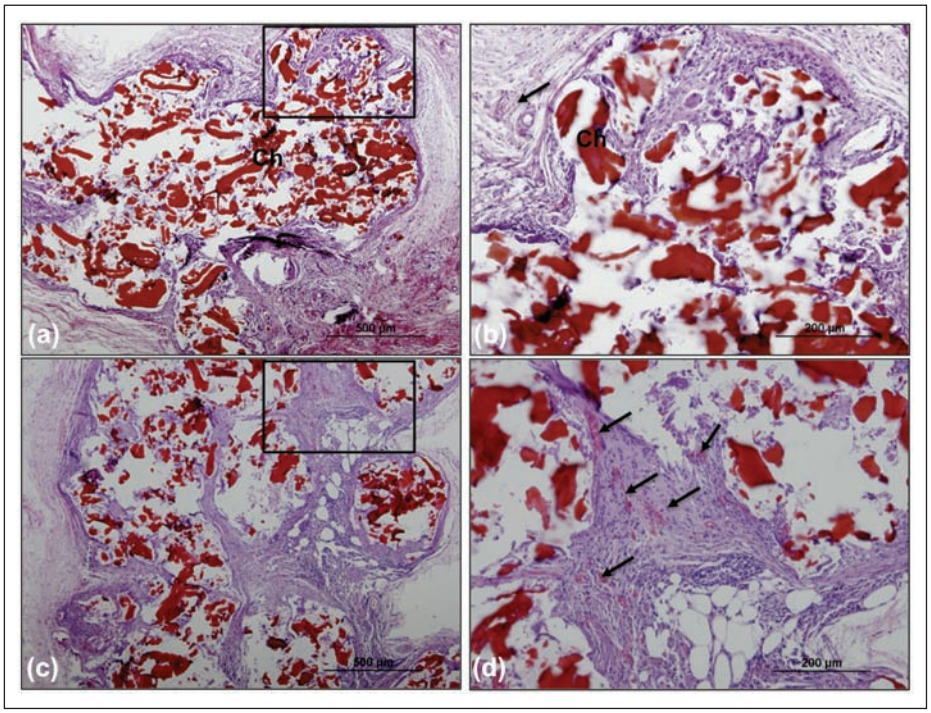

Figure 7. Representative H&E stained histological sections of tissues surrounding chitosan-based implants after (a and b) 6 weeks and (c and d) 12 weeks of subcutaneous implantation in Wistar rats; (b) and (d) represent the magnified sections of selected areas (square) of (a) and (b), respectively. Black arrows point to new blood vessels.
H&E: hematoxylin and eosin; Ch: chitosan.

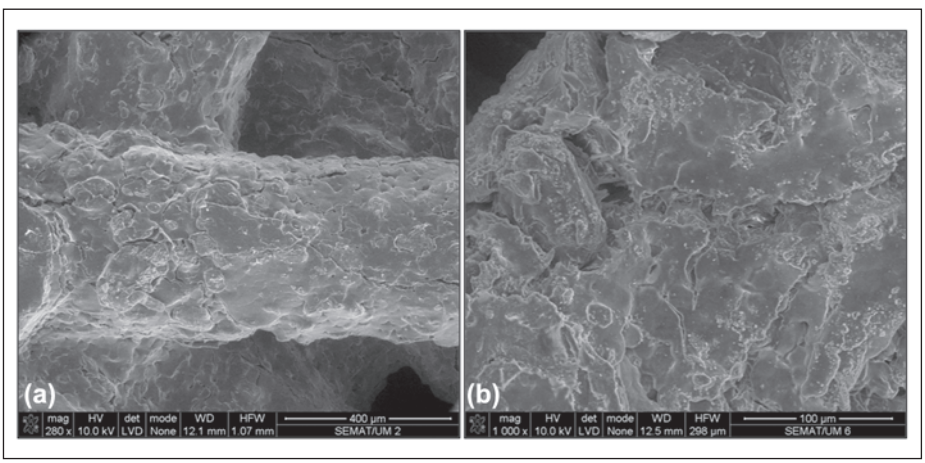

Figure 8. (a and b) SEM micrographs of chitosan–poly(butylene succinate) scaffolds after 12 weeks of in vivo implantation.
SEM: scanning electron microscopy.

implantation, the structural integrity of the scaffolds was retained. It should be noted the difference in kinetics of biodegradation in vitro and in vivo. The implanted scaffolds displayed a normal to mild tissue response, with the development of chronic inflammatory response and FBR.

Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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