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Biomaterials 26 (2005) 4606–4615
Biomaterials
www.elsevier.com/locate/biomaterials

Electrospun degradable polyesterurethane membranes: potential scaffolds for skeletal muscle tissue engineering

Stefania A. Riboldi[a], Maurilio Sampaolesi[b], Peter Neuenschwander[c], Giulio Cossu[b], Sara Mantero[a,*]

[a]*Department of Bioengineering, Politecnico di Milano, Piazza Leonardo da Vinci, 32, 20133 Milano, Italy*
[b]*SCRI Stem Cell Research Institute, San Raffaele Scientific Institute, 20132 Milano, Italy*
[c]*Department of Materials—Polymers, ETH Zurich, 8092 Zürich, Switzerland*

Received 11 June 2004; accepted 15 November 2004
Available online 7 January 2005

Abstract

Skeletal muscle tissue engineering represents an attractive approach to overcome problems associated with autologous transfer of muscle tissue and provides a valid alternative in muscle regeneration enhancement. The aim of this study was to investigate the suitability, as scaffold for skeletal muscle tissue engineering, of a known biodegradable block copolymer (DegraPol®) processed by electrospinning in the novel form of microfibrous membranes. Scaffolds were characterized with reference to their morphological, degradative and mechanical properties. Subsequently, cell viability, adhesion and differentiation on coated and uncoated DegraPol® slides were investigated using line cells (C2C12 and L6) and primary human satellite cells (HSCs). The membranes exhibited absence of toxic residuals and satisfactory mechanical properties (linear elastic behavior up to 10% deformation, E modulus in the order of magnitude of MPa). A promising cellular response was also found in preliminary experiments: both line cells and HSCs adhered, proliferated and fused on differently coated electrospun membranes. Positive staining for myosin heavy chain expression indicated that differentiation of C2C12 multinucleated cells occurred within the porous elastomeric substrate. Together the results of this study provide significant evidence of the suitability of electrospun DegraPol® membranes as scaffolds for skeletal muscle tissue engineering and that they represent a promising alternative to scaffolds currently used in this field.

Keywords: Block polyesterurethane; Electrospinning; Skeletal muscle tissue engineering; Satellite cell; Myoblast; Myotube

1. Introduction

Skeletal muscle, which is responsible for control of voluntary movement and maintenance of structural contours of the body, may be injured by exposure to myotoxic agents, such as bupivacaine or lidocaine; sharp or blunt trauma, such as punctures or contusions; ischemia, occurring with transplantation; exposure to excessively hot or cold temperatures [1]. Skeletal muscle tissue function is also affected by primary myopathies, which are characterized by a progressive wasting of skeletal muscle tissue that leads to deterioration of movements and, in the most severe cases, such as in Duchenne's Muscular Dystrophy (DMD), to complete paralysis and death [2,3].

Mature skeletal muscle is predominantly comprised of multinucleated, post-mitotic fibers that do not regenerate when damaged. Nevertheless, a population of quiescent myogenic progenitors, satellite cells, are capable of regeneration and compose 1–5% of the total nuclei of a mature muscle [4]. Satellite cells, sequestered between the sarcolemma and the individual mature muscle fibers, normally do not divide but can be induced to proliferate in response to specific local factors; they can migrate through the basal lamina sheets to enter the

*Corresponding author. Tel.: +39 2 23993376; fax: +39 2 23993360.
E-mail address: sara.mantero@polimi.it (S. Mantero).

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doi:10.1016/j.biomaterials.2004.11.035

injured area, where they fuse with pre-existing, damaged fibers or fuse to form new myotubes [5]. Once the proliferation potential of satellite cells is exhausted, there is no further regeneration and skeletal muscle is replaced by connective tissue. When muscle structure is irreversibly compromised or individual muscles (or part of them) have been ablated by surgical procedures or major injuries, the perspective of engineering new muscle fibers via satellite cells becomes an attractive though difficult goal.

Engineering muscle constructs in vitro would overcome problems associated with autologous transfer of muscle tissue and provide a valid alternative for tissue replacement in the enhancement of muscle regeneration. In addition, advantages of a readily available, non-immunogenic muscle for reconstruction include elimination of donor site morbidity and reduction of operative time and length of rehabilitation [5].

Many attempts have been made so far to reconstruct skeletal muscle tissue in vitro [6–15]; together the results of these studies suggest that is indeed feasible to engineer bioartificial muscles; nonetheless, with current technology, tissue-engineered skeletal muscle analogues are far from being a clinical reality. Morphologically, they fall short of actual skeletal muscle in many respects, including small-diameter myofibers, low myofiber organization and excessive extracellular matrix content. Since myofibers need to be packed parallel to each other to generate sufficient force for contraction [10,16], the lack of structural organization in engineered constructs results in too little active force to make them useful in clinical applications.

A key factor to overcome these problems is the design of appropriate scaffolds able to support cell fusion and the formation of long, continuous muscle fibers, which are essential to the adult phenotype of muscle. In accordance with the established "contact guidance" theory, in fact, cell proliferation and differentiation can be directed in preferential directions associated with chemical, structural and/or mechanical properties of the substratum [17,18].

In this context, the recent tendency to develop microfibrous polymeric scaffolds, in most cases made of classic degradable polyesters (PLA, PGA and copolymers), revealed promising results and succeeded in driving myofiber development and orientation along the preferential direction of the scaffold fibers [17,19-20]; nevertheless, the majority of these scaffolds suffer from some disadvantages, mainly with respect to their high tensile modulus (in the order of magnitude of GPa) and low-yield elongation (3–4%) [21–24]. The relative inflexibility and the limited possibility to modulate the mechanical properties of the cited polymers make them hardly compatible with the widespread "dynamic culture approach" in bioreactors, based on the hypothesis that contractile functional skeletal muscle tissue will fully develop in vitro only if subjected to mechanical strains that prevail during normal in vivo organogenesis and growth [6–10].

In order to overcome the above cited problems, we propose the use of DegraPol®, a degradable block polyesterurethane, consisting of crystallizable blocks of poly((R)-3-hydroxybutyric acid)-diol and blocks of poly(ε-caprolactone-*co*-glycolide)-diol linked with a diisocyanate. The use of DegraPol® as scaffold for tissue engineering has been investigated for a long time: now there is significant evidence of its in vitro [25–27] and in vivo [27,28] biocompatibility properties, as well as of its elastomeric behavior [29]. The results of these studies, together with the possibility to process the polymer by electrospinning in the form of microfibrous membranes, suggest that DegraPol® hold promises to be used also as scaffold for skeletal muscle tissue engineering.

Hence, in this study electrospun DegraPol® membranes were characterized with reference to their morphological, degradative and mechanical properties. Subsequently, we investigated the behavior of different myogenic cell types (C2C12, L6 and primary human satellite cells (HSCs)) in response to the scaffolds, either uncoated or coated with different proteins (Matrigel®, fibronectin and collagen).

2. Materials and methods

2.1. DegraPol® electrospun membranes

DegraPol® scaffolds were manufactured by electrospinning after solving the block copolymer with chloroform (30 wt%): electrospinning voltage (18 kV) was applied with a high-voltage supply between a needle and a rotating cylindrical collector. As-spun membranes were dried under vacuum at room temperature. The membranes, about 100 μm thick, can be roughly described as reticulated meshes of randomly or partially ordered fibers (about 10 μm in diameter) fixed at intersecting points. Before cell culture, 13 mm diameter DegraPol® slides were cut and sterilized by immersion in 70% (v/v) ethanol overnight and allowed to dry at room temperature in a sterile hood.

2.2. Scaffold characterization

The morphology of DegraPol® scaffolds was examined by means of a scanning electron microscope (SEM): samples were sputter coated with gold (Emitech K550, 4 min, 20 mA, 10^{-1} mbar) before examination under a Stereoscan S360 microscope (Cambridge Instruments) at an accelerating voltage of 15 keV.

As to the polymer degradation profile, in vitro degradation experiments were performed as follows:

electrospun DegraPol® membranes were bathed in buffered aqueous solution and kept at pH = 7 and 37 °C for 175 days. The reduction of the average molecular weight (M_p and M_w) was monitored via GPC (Viscotek TDA, Triple Detector Array) at days 0, 3, 7, 21, 42, 84 and 175 using tetrahydrofurane as eluting solvent.

Scaffolds were also evaluated for stress–strain response in the preferential direction of orientation of fibers by means of an electromechanical testing machine (M1000E, Mecmesin Limited) equipped with a 10 N load cell. Briefly, electrospun DegraPol® samples were cut in dog-bone shape 4 mm wide (W) and their thickness (s) was measured by means of a digital micrometer (Mitutoyo America Corporation). After three preconditioning cycles up to 10% deformation, each sample was tested at rupture at 6 mm/min. Stress σ ($\sigma = F/(Ws)$) was plotted as a function of ε ($\varepsilon = \Delta l/l_0 \times 100$), while ultimate stress σ_{max}, elastic modulus E (slope of the $\sigma\varepsilon$ plot between 0 and 10% deformation) and ultimate percent deformation (ε_{max}, ε corresponding to σ_{max}) were calculated (mean values and standard deviations, $n = 4$).

2.3. Cell culture

Three cell types were cultured in order to evaluate their different responses on DegraPol® membranes. We used C2C12 (murine myoblast cell line, CRL-1772, ATCC, Rockville, MD, USA), L6 (rat myoblast cell line, CRL-1458, ATCC, Rockville, MD, USA) and primary HSCs (biopsy from 38-yr old female) isolated as previously described [30]. Cells were plated in 75 cm^2 tissue-culture flasks and cultured in DMEM (Dulbecco's Modified Eagle's Medium, Gibco Laboratories Inc., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 4 mM L-glutamine, adjusted to contain 1.5 g/l sodium bicarbonate and 4.5 g/l glucose, 100 IU/ml penicillin and 100 μg/ml streptomycin. Flasks were incubated at 37 °C in a humidified 5% CO_2 atmosphere. Cells were harvested with 2 ml trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 10 min and immediately re-suspended in complete fresh medium to stop trypsinization; cell number was subsequently estimated using a Burker chamber.

2.4. Cell viability (cytotoxicity of eluates)

Prior to cell–DegraPol® contact, an MTT test for cytotoxicity of eluates was performed in order to verify the absence of toxic residuals in the scaffolds as a result of the peculiar processing technique. Briefly, sterile DegraPol® slides were eluted for 7 days in 1 ml complete culture medium (at 37 °C in a humidified 5% CO_2 atmosphere). Eluate withdrawals were performed at days 1, 3 and 7. Contemporaneously, C2C12 seeded in 96-well dishes (2×10^3 cells/well) were grown in complete medium for 24 h. After that, culture medium was replaced with DegraPol® eluates (100 μl/well) and cells were further on incubated for 24 h. Cell viability was assessed via colorimetric assay (MTT test): absorbance values were measured using a Tecan GENios microplate reader (Tecan Group Ltd., Maennedorf, Switzerland) at a wavelength of 570 nm (reference wavelength 630 nm). Medium incubated on tissue culture polystyrene (TCPS) served as control.

2.5. Adhesion and proliferation assessment

In order to evaluate cell adhesion and proliferation on DegraPol® membranes, C2C12, L6 and primary HSCs were cultured on the material, either coated or uncoated, using TCPS as a control. Briefly, after sterilization and drying, scaffolds were treated with 500 μl/well of different coating solutions: 10% collagen solution (Collagen type I, Sigma-Aldrich, St. Louis, MO, USA), concentrated serum fibronectin (Sigma-Aldrich, St. Louis, MO, USA), Matrigel® (Becton Dickinson and Company, Franklin Lakes, NJ, USA) at a concentration of 130 μg/ml and Matrigel® at 13 μg/ml. Uncoated DegraPol® slides and polystyrene wells were used as controls. C2C12, L6 and primary HSCs were seeded at a density of 10^5 cells/well; complete medium was added (500 μl/well) before incubating cells for 3 days at 37 °C in a humidified 5% CO_2 atmosphere. Medium was replaced after 48 h. For quantitative assessment of cellular proliferation, the above-mentioned experiment was carried out in triplicate and the number of cells on test substrata was assessed at day 1, 2 and 3 as follows: after aspiring culture medium and gently rinsing with PBS, cells were fixed with 4% formaldehyde for 5 min, stained with propidium iodine and counted under a Nikon Eclipse TE 200 microscope equipped with a DXM 1200 Nikon camera. Mean values and standard deviations were calculated.

2.6. Cell differentiation

The last experiment was carried out in order to evaluate DegraPol® capability of inducing cell fusion and differentiation into myotubes. Sterile DegraPol® slides were coated with Matrigel® 130 μg/ml by solution deposition; uncoated DegraPol® slides and coated/uncoated polystyrene wells were used as controls. GFP-C2C12, myogenic line cells previously labelled with a lentiviral vector expressing green fluorescent protein [3], and HSCs were seeded in duplicate at densities of 10^5 C2C12 cells/well and 2×10^5 HSCs/well; additional complete medium was added (1 ml/well) before incubating cells at 37 °C in a 5% CO_2 humidified atmosphere. Cells were cultured on DegraPol® slides and on control surfaces in complete growth medium.

Differentiation medium (with 2% HS—Horse Serum) was added at day 4 in order to induce differentiation into myotubes. The duration of the whole experiment was 7 days, while medium was changed every 2 days.

Samples were fixed in 2% paraformaldheyde for 5 min at room temperature, rinsed three times with PBS, permeabilized for 5 min at room temperature by addition of a buffer containing 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS, finally washed two times with PBS and blocked with PBS containing 5% donkey serum. The fixed cells were subsequently incubated for 2 h at 37 °C with a rabbit polyclonal antibody against skeletal myosin heavy chain (MHC) 1:250 in PBS. Following two rinses with PBS, DegraPol® specimens were incubated for 1 h with a solution containing FITC conjugated anti-IgG rabbit-in-donkey (1:100), 1% rhodamine–phalloidin for F-actin staining and 0.05% Hoechst as a nuclear marker (Sigma-Aldrich, St. Louis, MO, USA). Images were collected using a Spectral Confocal system (Leica TCS SP2SP AOBS, Heidelberg, Germany). The specimen morphology was investigated by SEM analysis. Briefly, cells were washed with deionized water and dehydrated by graded ethanol changes (from 30% to 100% ethanol in deionized water) and dried with xylene. Samples were subsequently gold sputtered in vacuum (Emitech K550, 4 min, 20 mA, 10^{-1} mbar) before examination under a Stereoscan S360 microscope (Cambridge Instruments) at an accelerating voltage of 15 keV.

3. Results

3.1. Scaffold characterization

SEM micrographs of DegraPol® samples are shown in Fig. 1a and b. The three-dimensional fibrous mesh (about 100 μm thick) consists of fibers about 10 μm in diameter with a fiber-to-fiber distance of about 10 μm. A slight preferential orientation of fibers is detectable (due to the rotational direction of the collector during electrospinning processing), while interconnections between fibers provide voids, so that an open-pored structure is formed.

Fig. 2 shows the reduction of DegraPol® molecular weight in buffered aqueous solution at 37 °C. After 4 weeks of hydration, the average M_p of the polymer has dropped of about 45% of the initial value. At week 12, this value has decreased further, approaching 25% of the starting value. Within 25 weeks, the average M_p value is below 10000. Data obtained from Fig. 2 are consistent with the ones found in literature [28]; relatively large differences in starting molecular weight level off after advanced degradation.

As shown in Fig. 3, the typical stress–strain response of electrospun DegraPol® samples exhibits a linear elastic behavior up to 10% deformation. Table 1 gives mean values (μ) and standard deviations (σ) of the membrane tensile properties ($n = 4$): tensile strength (σ_{max} (MPa)), tensile modulus in the 0–10% deformation range (E (MPa)) and deformation at break (ε_{max} (%)). Samples show tensile modulus E in the order of magnitude of MPa (10.15 ± 0.69 MPa) and deformation at break of about 200% (220.40 ± 57.09%).

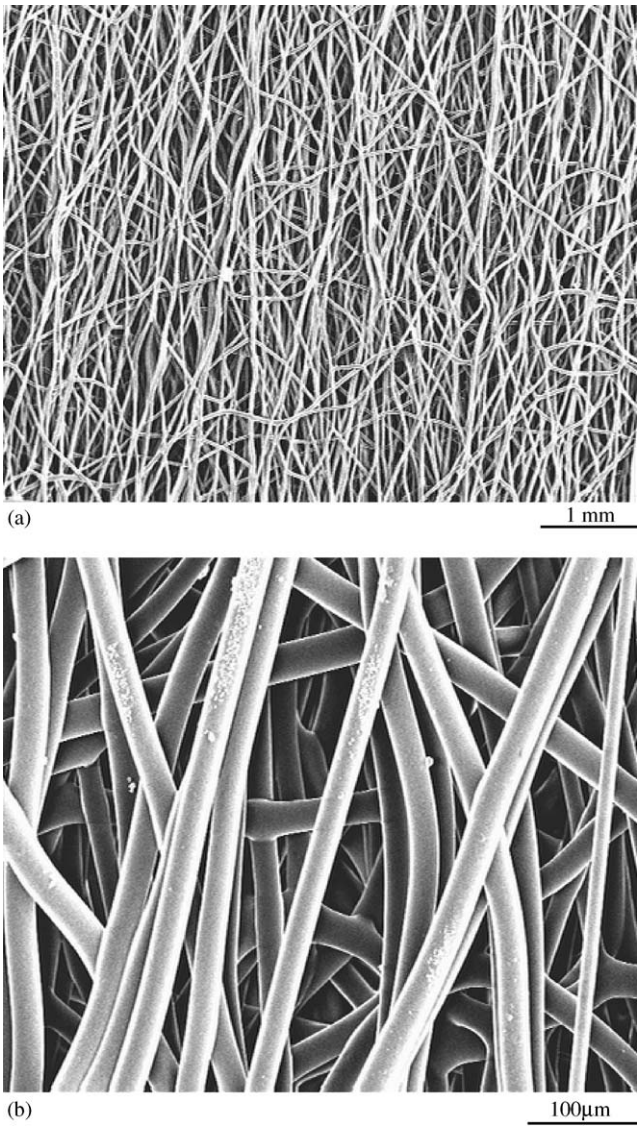

Fig. 1. (a,b) SEM micrographs of DegraPol® membranes. DegraPol® samples were sputter coated with gold (Emitech K550, 4 min, 20 mA, 10^{-1} mbar) before examination under a Stereoscan S360 microscope (Cambridge Instruments) at an accelerating voltage of 15 keV. A slight preferential orientation of fibers is detectable, while interconnections between fibers provide voids so that an open-pored structure is formed.

3.2. Cell viability (cytotoxicity of eluates)

Cell viability after contact with DegraPol® eluates was estimated via colorimetric assay (MTT test) by

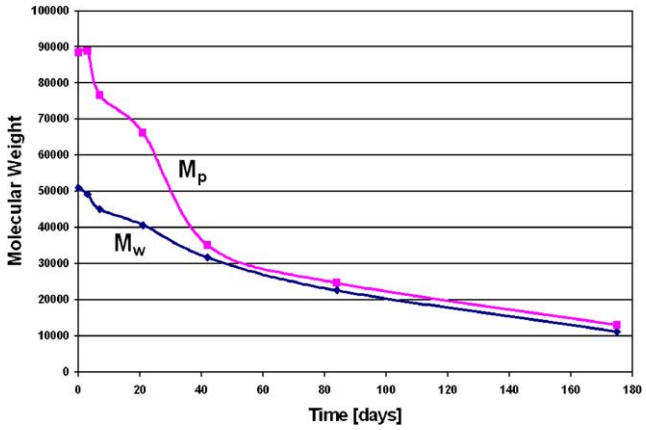

Fig. 2. DegraPol® degradation in buffered aqueous solution at 37 °C. After 4 weeks of hydration, the average M_p of the polymer has dropped of about 45% of the initial value. At 12 weeks, this value has decreased further, approaching 25% of the starting value. Within 25 weeks, the average M_p value is below 10000.

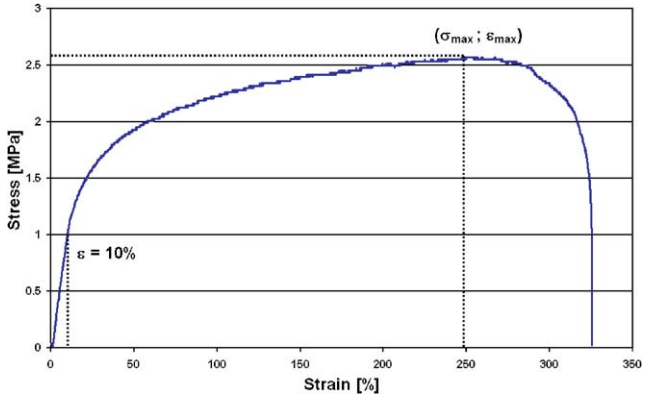

Fig. 3. Stress–strain response representative of a typical dog-bone electrospun DegraPol® sample. Tensile tests were performed at 6 mm/min, following three preconditioning cycles. The curve exhibits a linear elastic behavior in the 0–10% deformation range and deformation at break higher than 200%.

Table 1
Tensile properties of electrospun DegraPol® samples

	Tensile strength σ_{max} (MPa)	Tensile modulus E (MPa)	Elongation at break ε_{max} (%)
μ	2.52	10.15	220.40
σ	0.17	0.69	57.09

Mean values (μ) and standard deviations (σ) of the membrane tensile properties ($n = 4$) are given: tensile strength (σ_{max} (MPa)), tensile modulus in the 0–10% deformation range (E (MPa)) and deformation at break (ε_{max} (%), ε corresponding to σ_{max}).

measuring the absorbance of the solution at different time points. Fig. 4 shows absorbance values (mean and standard deviation, $n = 9$) relative to DegraPol® eluates at days 1, 3 and 7. TCPS eluates were used as controls.

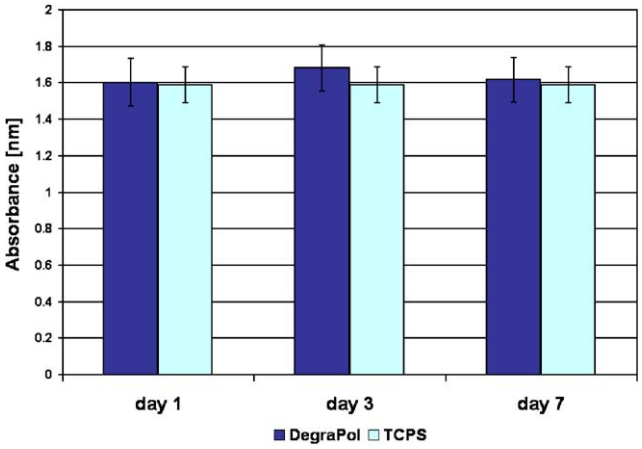

Fig. 4. Cell viability after contact with DegraPol® eluates. An MTT test was carried out culturing C2C12 line cells in the presence of DegraPol® eluates, withdrawn after 1, 3 and 7 days of elution. TCPS eluates were used as controls. DegraPol® eluates give absorbance values comparable to controls, showing that no toxic residuals were present in the membranes as a result of electrospinning processing.

Histograms reveal that, at all the time points, the viability of cells after contact with DegraPol® eluates is comparable with controls.

3.3. Adhesion and proliferation assessment

Cell proliferation on coated and uncoated DegraPol® slides and on TCPS was assessed by counting the number of cells at days 1–3 after seeding. Optical observations performed at day 3 (Fig. 5a) show that C2C12 murine cells adhered on Matrigel® coated DegraPol® slides as well as on TCPS (Fig. 5a, upper lane), especially when Matrigel® was added at high concentration (130 μg/ml). Good results were also obtained in the case of collagen-coated DegraPol® slides and with a lower concentration of Matrigel® (13 μg/ml). After the same period of culture, L6 rat myogenic cells showed similar behavior on DegraPol® slides, except for a better adhesion to collagen-coated DegraPol® membranes (Fig. 5a, middle lane). As expected, HSCs showed a lower adhesion on all tested materials with respect to cell lines, but uncoated and Matrigel®-coated DegraPol® slides favored a cell adhesion degree comparable to polystyrene controls (Fig. 5a, lower lane). Quantitative data with reference to uncoated DegraPol® slides seeded with different cell types are shown in Fig. 5b; polystyrene is shown as a control.

3.4. Cell differentiation

After 3 days of culture in differentiation medium, immunofluorescence microscopy of C2C12 cells grown on Matrigel®-coated DegraPol® membranes showed the presence of elongated, multinucleated, myosin

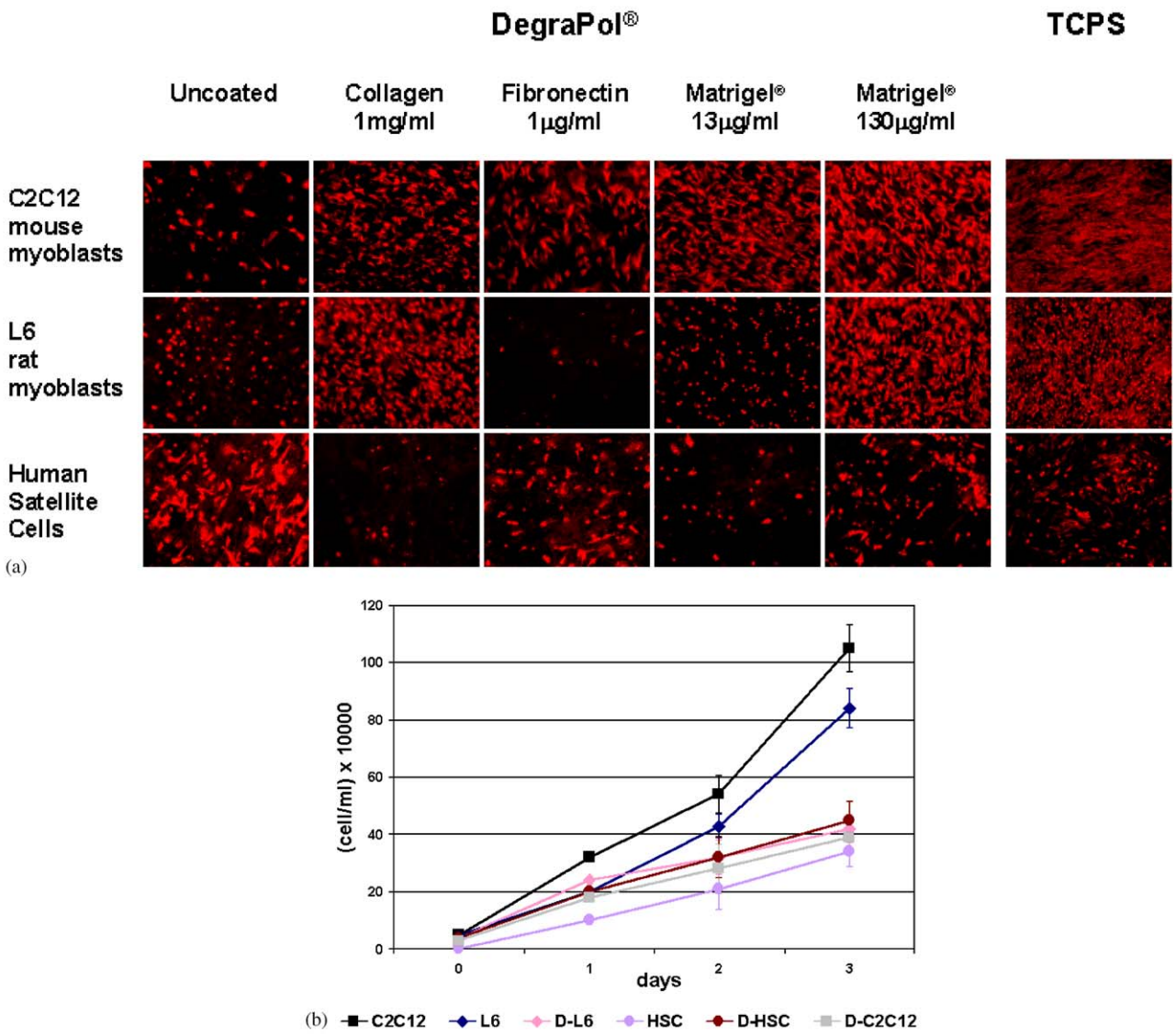

Fig. 5. Cell adhesion assessment on coated and uncoated DegraPol® slides. Line myoblasts (C2C12 and L6) and primary HSCs were cultured for 3 days on coated (coatings: collagen, fibronectin and Matrigel® at different concentrations) and uncoated DegraPol® membranes. Polystyrene (TCPS) was used as a control. (a) Cells labelled with propidium iodine after 3 days culturing. (b) Growth curves with reference to uncoated DegraPol® slides (D-L6, D-C2C12 and D-HSC) and polystyrene wells as controls (L6, C2C12 and HSC).

expressing myotubes. Fig. 6A, A′ and A″ represent myoblast nuclei (stained with Hoechst), F-actin (stained with rhodamine–phalloidin) and FITC conjugated antibody for MHC, respectively. A clear tendency of C2C12 myotubes to align along the preferential direction of the scaffold fibers is detectable. HSCs cultured on Matrigel®-coated DegraPol® scaffolds fused into polynucleated F-actin-expressing cells, but no myosin expression was detectable. Images of HSCs are shown in Fig. 6B, B′ and B″. Fig. 6B″ shows the result of SEM analysis of a human multinucleated myocyte adhering on DegraPol® fibers with cytoplasmatic spreading inside DegraPol® porosity. Fig. 6B shows nuclear markers and Fig. 6B′ F-actin expression relative to that human myocyte.

4. Discussion

This work investigates on the potential use of electrospun DegraPol® membranes as fibrous scaffolds for skeletal muscle tissue engineering. In order to evaluate their suitability for this specific application, scaffolds were characterized with reference to their morphological, degradative and mechanical properties. Subsequently, cell viability, adhesion and differentiation on coated and uncoated DegraPol® slides were investigated using line cells (C2C12 and L6) and primary HSCs. Before discussing the significance of our results, we first address some issues related to skeletal muscle tissue engineering and to DegraPol® relevance in the tissue engineering field.

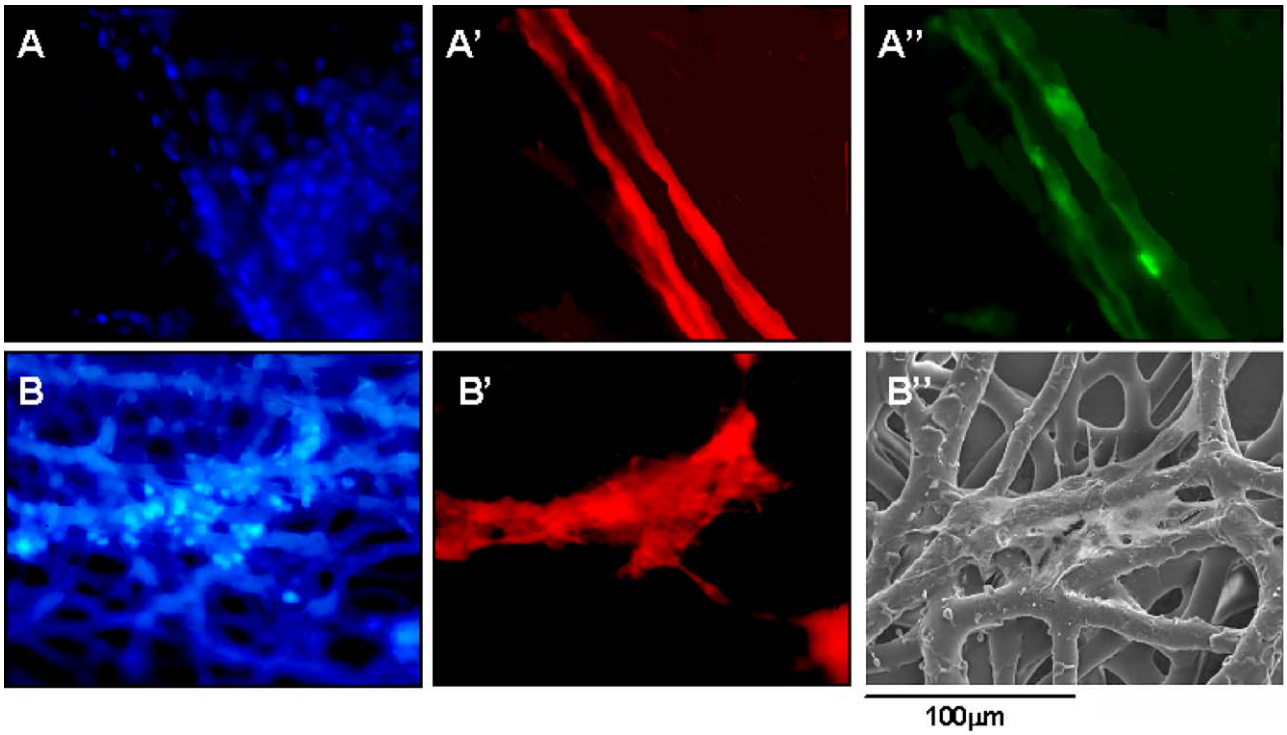

Fig. 6. Myoblast differentiation on Matrigel®-coated DegraPol® slides in differentiation medium (2% HS). Upper lane: C2C12 cells. Hoechst-stained myoblast nuclei (A), F-actin stained with rhodamine-phalloidin (A′), FITC-conjugated antibody for MHC (A″). Lower lane: primary HSCs fusing into a myotube. Hoechst-stained nuclei (B), F-actin expression (B′) and SEM micrograph (B″).

Many attempts have been made so far to reconstruct skeletal muscle tissue in vitro. Some of these focus on developing self-organizing constructs, employing no artificial scaffolds for the contractile segment of the engineered muscle [6,7]; other groups directed their efforts toward seeding cells on natural or synthetic biodegradable substrates (collagen matrices [8–10], polyglycolic acid meshes [11–13], silanized glass scaffolds [14], commercially available polyurethanes [15]) in order to induce three-dimensional organization of myoblasts. The results of these studies suggest that it is indeed feasible to engineer bio-artificial muscles; nonetheless, with current technology, tissue-engineered skeletal muscle analogues are far from being a clinical reality. Morphologically, they fall short of actual skeletal muscle in respect to small-diameter myofibers, low myofiber organization and excessive extracellular matrix content. Since myofibers need to be packed parallel to each other to generate sufficient force for contraction [10,16], the lack of structural organization in engineered constructs results in too little active force to make them useful in clinical applications.

Two key factors to solve these issues are worth mentioning: (1) the design of appropriate scaffolds, able to direct cell proliferation and differentiation in preferential directions associated with chemical, structural and/or mechanical properties of the substratum (contact guidance theory) [14,17-18]; (2) the dynamic culture approach, aiming at favoring the development of a native-resembling tissue by mimicking the environmental conditions that prevail during normal in vivo organogenesis and growth.

As far as the scaffold morphology is concerned, promising results have been found using microfibrous polymeric scaffolds made of classical degradable polyesters (PLA, PGA and copolymers), which were shown to direct myofiber orientation and tissue growth in a highly unidirectional way [17,19-20]. Nevertheless, the limited elastic character exhibited by the above-cited scaffolds makes them hardly applicable in dynamic culture experiments performed in bioreactors: they usually exhibit excessively high tensile modulus (in the order of magnitude of 1 GPa) and low-yield elongation (3–4%) [21–24].

In this context, we address a huge potential of electrospun DegraPol® scaffolds for application in the field of skeletal muscle tissue engineering. DegraPol® is a degradable block polyesterurethane, consisting of crystallizable blocks of poly((R)-3-hydroxybutyric acid)-diol and blocks of poly(ε-caprolactone-*co*-glycolide)-diol linked with a diisocyanate. Being a block copolymer, DegraPol® combines the advantages of traditional polyesters with high processability and marked elasticity properties [29]. The use of DegraPol® foams and porous membranes as scaffolds for tissue engineering has been investigated for a long time; now

there is significant evidence of DegraPol® in vitro [25–27] and in vivo [27,28] biocompatibility properties. Saad et al. studied the response of in vitro cultured fibroblasts, macrophages and osteoblasts to the crystalline domain of the material [25]. They report that phagocytosis of the crystalline segments causes dose-dependent cell activation, cell damage and cell death in macrophages but not in fibroblasts. Their studies moreover indicate that DegraPol® exhibits good cell compatibility and does not induce cytotoxic effects in osteoblasts and condrocytes [25–27]. Saad et al. also performed subcutaneous implantations of polymer foils in rats; the results of these in vivo experiments showed that after 2 months of implantation the thickness of the capsule was lower than 30 μm [27]. Borkenhagen [28] describes the in vivo performances of DegraPol® tubular structure used as nerve guidance channel, showing that the inflammatory reaction associated with polymer degradation does not interfere with nerve regeneration process. Recently, DegraPol® scaffolds were manufactured by electrospinning also in the novel form of microfibrous membranes. There is significant potential for using electrospinning technology to manufacture scaffolds for tissue engineering applications: the method is relatively simple, highly controllable, reproducible, productive and it is able to co-spin polymers with various additives tuning the function of the scaffold toward selected cell type [17].

In this study we addressed evidence of the suitability of electrospun DegraPol® scaffolds for application in the field of skeletal muscle tissue engineering, especially due to their satisfactory mechanical properties and to the promising cellular response in preliminary adhesion and differentiation experiments. A brief discussion of the significance of the above-cited results is given below.

As to the membrane mechanical properties, we pointed out that they exhibit linear elastic behavior in the 0–10% deformation range (Fig. 3) and deformations at break higher than 200% (Table 1). Such characteristics enable DegraPol® to be applied both in dynamic culture experiments and for tissue engineering of soft tissues, thus overcoming problems related to the relative inflexibility of traditional degradable polyester scaffolds. Matching the mechanical properties of a scaffold to the graft environment is critically important so that progression of tissue healing is not limited by mechanical failure of the scaffold prior to successful tissue regeneration [31]. While traditional degradable polyesters exhibit elastic modulus values in the order of magnitude of gigapascals (well-suited for tissue engineering of hard tissues), electrospun DegraPol® membranes show E values around 10 MPa (Table 1), better matching the range of elasticity of soft tissues such as skeletal muscle. The high elasticity of our scaffolds is due to both the elasticity of the fiber material and the reticulated structure of the membrane.

With respect to cell response to electrospun DegraPol® membranes, preliminary experiments showed a favorable cell/scaffold interaction. Here is a brief summary of the results.

C2C12 viability after contact with DegraPol® eluates confirms the absence of toxic residuals within the polymeric matrix (Fig. 4); this fact allows to rule out the release of toxic substances (i.e. chloroform) in case present in the membranes due to electrospinning processing.

As to cell adhesion, C2C12, L6 and primary HSCs showed to adhere and proliferate on electrospun DegraPol® scaffolds (Fig. 5a), but cell adhesion (in comparison to polystyrene controls) varied among cell species according to the membrane coating. The need to coat the scaffolds arose from the known principle that cell adhesion processes are mediated by integrins, transmembrane receptors that selectively bind ligand domains on extracellular matrix proteins (collagen, fibronectin, etc.). Since different cell species express disparate integrin types, we believe that differences in cell behavior on DegraPol® are due to different chemical affinities between integrin receptors and the ligand binding domains of different protein coatings. Quantitative and comprehensive experiments concerning this aspect will be the subject of future studies.

As far as the last experiment is concerned, Hoechst nuclear staining demonstrated that cell fusion had taken place on Matrigel®-coated DegraPol® membranes both in the case of C2C12 (Fig. 6A) and with primary HSCs (Fig. 6B). Positive staining for MHC expression (FITC label) indicated that differentiation of C2C12 multinucleated cells occurred within the porous elastomeric substrate (Fig. 6B″). However, since we did not perform any gene expression analysis in this work, further studies are needed in order to evaluate the expression of specific muscle markers (such as myoglobin and desmin) and to investigate the nature of myofibers. With respect to the absence of myosin expression in satellite cells, we believe that a possible reason could be the fact that the whole experiment was carried out in the presence of Matrigel® coating, while Fig. 5 shows that HSCs more favorably respond to uncoated DegraPol® membranes. However, since differentiation on a substrate is usually a critical issue, particularly using precursor cells such as satellite cells and myoblasts, we think that the formation of multinucleated HSCs and C2C12 myotubes on the membranes indicates DegraPol® as a promising scaffold for skeletal muscle tissue engineering.

Along with the above-cited aspects, other noteworthy features shown by electrospun DegraPol® scaffolds are the following. DegraPol® degradation time appeared to be appropriate both to support in vitro tissue development and in vivo tissue remodeling: the polymer is degradable by hydrolysis and the molecular weight has

been shown to decade below 10,000 in about 180 days (Fig. 2); tensile strength measurements at different time points during membrane degradation confirmed this hypothesis (data not shown). Electrospun membranes also exhibited suitable morphological properties for myoblasts adhesion and organization; a clear tendency of C2C12 myotubes to align along the preferential direction of the scaffold fibers was detected (Fig. 6A′ and A″). With respect to this last issue, Neumann et al. [17] found that C2C12 cells cultured on arrays of polypropylene fibers 10–15 μm in diameter formed up to 50μm-thick layers of longitudinally aligned cells. They also found that the optimal distance between fibers in order to favor the formation of complete cell sheets is 55 μm or less. Electrospun DegraPol® membranes fairly well suit this requirement (see Fig. 1a and b), though a quantitative image analysis was not performed in this study.

On the basis of these findings, we believe there is significant evidence of the suitability of electrospun DegraPol® membranes as scaffolds for skeletal muscle tissue engineering and that they represent a promising alternative to scaffolds currently used in this field. A lot of work, however, remains to be done in order to tune the scaffold characteristics to the specific application: in this sense, an encouraging feature of our scaffolds is the possibility to vary independently their surface morphology, elastic properties and degradation rate. The chance of controlling the overall degree of orientation of fibers by simply modulating one processing parameter, for example, is useful to produce scaffolds able to direct myoblasts growth along a preferential direction, inducing and enhancing cell fusion and differentiation into myotubes.

5. Conclusion

In this study we collected evidences of the suitability of electrospun DegraPol® membranes for use as scaffolds for skeletal muscle tissue engineering: they especially showed satisfactory mechanical properties and promising cellular response in preliminary adhesion and differentiation experiments. Further studies will be carried out to gain a deeper knowledge in cell adhesion and differentiation mechanisms on DegraPol®, particularly with reference to (a) the need to coat the scaffolds with ECM proteins and (b) to the expression of specific muscle markers. In addition, efforts are being made in order to tune the scaffolds characteristics to the specific application: membranes with high degree of orientation of fibers have been manufactured with the aim to enhance cell alignment and fusion and the formation of long, continuous muscle fibers, which are essential to the adult phenotype of muscle.

Acknowledgments

We thank Paolo Mognol and Marc Simonet for their support in producing and processing DegraPol® scaffolds.

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