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Research Article

Preparation and Characterization of an Advanced Medical Device for Bone Regeneration

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Abstract. Tridimensional scaffolds can promote bone regeneration as a framework supporting the migration of cells from the surrounding tissue into the damaged tissue and as delivery systems for the controlled or prolonged release of cells, genes, and growth factors. The goal of the work was to obtain an advanced medical device for bone regeneration through coating a decellularized and deproteinized bone matrix of bovine origin with a biodegradable, biocompatible polymer, to improve the cell engraftment on the bone graft. The coating protocol was studied and set up to obtain a continuous and homogeneous polylactide-co-glycolide (PLGA) coating on the deproteinized bone matrix Orthoss® block without occluding pores and decreasing the scaffold porosity. The PLGA-coated scaffolds were characterized for their morphology and porosity. The effects of PLGA polymer coating on cell viability were assessed with the 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium assay. The polymer solution concentration and the number of polymeric layers were the main variables affecting coating efficiency and porosity of the original decellularized bone matrix. The designed polymer coating protocol did not affect the trabecular structure of the original decellularized bone matrix. The PLGA-coated decellularized bone matrix maintained the structural features, and it improved the ability in stimulating fibroblasts attachment and proliferation.

KEY WORDS: biodegradable polymers; bone regeneration; medical devices; polylactide-co-glycolide.

INTRODUCTION

Bone tissue can undergo serious degenerative problems due, i.e., to nonunion fractures or pathologies such as bone tumors. In some of these cases, advanced medical devices promoting the tissue regeneration can be a useful support to the natural self-organizing tissue regeneration. The recent scientific literature is rich in studies and reviews about consolidated and newly experimental techniques focused on bone regeneration, such as transplanting of autologous bone (autografting bone of the same patient), transplanting of allogenic bone (bone from a human cadaver), or of xenogenic bone (bone from animal source) (1). Polymers represent a further opportunity to set up substrates for bone tissue regeneration and to overcome drawbacks such as pain or risk of morbidity, involved in the cited techniques. For this reason, countless studies have been developed in these years involving polymeric scaffolds for bone tissue regeneration (2). The polymer choice is of outmost importance to achieve the substrate scaffold with the characteristics suitable to support and improve bone regeneration. The known general requirements for a scaffold substrate for tissue regeneration are: a) biocompatibility and absence of immunogenic reactions; b) tridimensional architecture with highly interconnected porous structure, c) surface bioactivity and, d) singular degradation rate to lead to the new tissue formation. Moreover a scaffold for bone regeneration should show appropriate mechanical properties, and structural anisotropy as it affects the mechanical behavior, cell orientation into the scaffold, and the deposition of extra-cellular matrix (3,4).

Orthoss® is a product registered as a class III medical device commercialized for human use (dental and orthopaedic); it is a unique bone graft substitute material with exceptional biofunctionality for filling and reconstruction of aseptic bone cavity and defects. The material is a decellularized and deproteinized bone of bovine origin. The manufacturing process adheres to the strict control measures and clinical documentation in accordance with a quality assurance system based on international standards (ISO 13485 and ISO 9001). The data of safety concerning the use in humans of the bone substitute of bovine origins, were provided by the manufacturing company at registration site of the product; they are also well documented by the scientific literature available on Orthoss® (5–10). Chemically speaking, Orthoss® is a matrix made of natural hydroxyapatite, the highly osteoconductive natural matrix possesses a topography which is very similar to

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the human bone. Orthoss® facilitates angiogenesis and migration of osteoblasts throughout the matrix, and when it is implanted, the matrix is structurally integrated into the surrounding bone and it is incorporated into the physiological remodeling process (8–10).

Orthoss® is an ideal alternative to autologous bone for filling of smaller defects. During reconstruction of larger defects, Orthoss® is well suited to be used in combination with autologous bone or as a carrier matrix in combination with bone marrow aspirate concentrate. The material is available on the market as spongious granules and as blocks of variable sizes that fit the different surgeons needs. As an example, Orthoss® block is ideal for indications as bone graft substitute in high tibial osteotomy, anterior crucial ligament revision, tibial plateau fracture, bone cysts, or arthrodesis diseases (10–15).

Orthoss® accomplishes all the above requirements of a suitable substrate for bone regeneration. Nevertheless, the coating with a biodegradable polymer could be advantageous for two reasons: a) to further improve cell seeding and proliferation on the scaffold; b) to encapsulate progenitor cells and osteoinductive factors into the polymer film producing a double-phase system with enhanced osteogenic, osteoinductive, and osteoconductive properties.

In fact, the *in vivo* application of scaffold has the advantage that all the components necessary for tissue regeneration, such as growth factors, are provided by the biological environment or possibly by bone marrow aspirate concentrate when it is combined to the autologous bone (16). However, different clinical answers to the regenerative therapy have been highlighted in young healthy patients with respect to old sick patients, suffering for example of diabetes or hyperlipidemia, and presenting lower regenerative potential. For the latter type of patients, the use of scaffolds with improved osteogenic, osteoinductive, and osteoconductive properties can be highly advantageous in a faster recovery from bone injury (17).

The goal of this preliminary work was to develop a protocol for coating Orthoss® block scaffold with a polymeric biodegradable film that subsequently could be used to encapsulate growth factors. In this study, the coating protocol was developed and optimized focusing on keeping or eventually improving the structural and functional properties of the scaffold.

The polymer selected for scaffold coating is polylactide-co-glycolide (PLGA). It is a biocompatible and biodegradable polymer approved for medical use in humans with several advantages: (1) it is a synthetic very versatile compound, its properties can be tailored as a function of the forecasted application; (2) it has been used for long time (almost 30 years) as suture in surgical operations with excellent results, and (3) it is either component of medical devices, used in orthopaedic surgery, and/or of several drug delivery systems on the market. Composites made of polylactic acid-calcium phosphates and polylactic acid-co-glycolic acid-calcium phosphates have seen widespread uses in orthopedic applications (18). Moreover, PLGA has been tested, with positive results, as injectable scaffolds to improve bone quality in osteoporotic female rats, in combination with collagen type I and bone mesenchimal stem cells (19). These reasons lead to the belief that PLGA is a safe and suitable polymer for use in tissue engineering.

In a previous work, of the same research group, Bioss® block were coated with polylactide (PLA) (20). The positive results achieved with the polyester coating led us to investigate the coating process to Orthoss® blocks. These scaffolds are bigger with respect to Bioss® blocks and are intended for orthopedic application while Bioss® blocks are suggested for dental use. Moreover, as the high hydrophobicity of PLA could results in few drawbacks in the coating of big blocks such as the Orthoss® ones, in the present work the biodegradable biocompatible polymer PLGA was investigated as the coating polymer. In fact, a more hydrophilic polymer such as PLGA, could lead to higher cell affinity improving cell attachment. Moreover, the PLGA degradation behaviour could be more suitable than PLA to the subsequent forecasted use of the polymer coating as drug delivery system (DDS), e.g., for growth factors or antibiotics. A detailed investigation was here performed on the coating process to optimize the process parameters in terms of polymer concentration and coating steps. The paper deepens aspect concerning the preparation and characterization of the medical device that can be of interests to scientists involved in issues related to biomaterials and scaffold fabrication techniques.

MATERIALS AND METHODS

Materials

Orthoss® block (block size, 2×2×1.3 cm) were provided by the manufacturer Geistlich-Pharma-CH-6110 Wohlusen, Switzerland, as commercially available.

PLGA, average of Mw 130 kDa, inherent viscosity (~0.5% (*w*/*v*) in $CHCl_3$ at 30°C), 0.7 dL/g was from Lakeshore Biomaterials, USA.

1,4-dioxane of analytical grade was from Sigma-Aldrich srl (Milano, Italy).

4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) was from Sigma-Aldrich srl (Milano, Italy).

Adult dermal fibroblast as primary cells were purchased from International PBI (Milan, Italy). The cells were cultured in DMEM containing 10% fetal bovine serum (FBS; Eu approved) and 1% antibiotic solution (100 U/mL penicillin, 100 μg/mL streptomycin, Sigma-Aldrich). After expansion, at 5th–6th passages, the cells were detached for the cell seeding experiments.

Methods

Scaffold Coating Process

The scaffold coating process was performed by soaking the Orthoss® blocks into a PLGA 1,4-dioxane solution. The process consisted in different steps as follows.

- Scaffold conditioning: the Orthoss® blocks were preliminary soaked in a 1,4-dioxane bath for 20 min to wet them and facilitate the subsequent diffusion of polymer solution into the trabecular structure. At the end of the 20 min of soaking, the scaffolds were withdrawn from 1,4-dioxane bath and dripped.
- Scaffold coating: the conditioned Orthoss® blocks were soaked in the PLGA 1,4-dioxane solution, and while soaking, they underwent subsequent cycles of depressurization/

pressurization to force the penetration of the polymer solution in the Orthoss® block pores.
– After this treatment, the scaffolds were withdrawn from the polymer solution and freeze dried.
– The coating cycle and the subsequent freezedrying cycle was repeated more than one time to obtain a multi-layered coating.

Figure 1 shows a scheme of a single cycle of the set up coating process. The following process parameters were investigated to optimize Orthoss® coating: polymer solution concentration (0, 4, 6, 7, and 10% (*w*/*v*)), soaking time of Orthoss® blocks in the polymer solution (30, 60, and 90 min), and number of coating cycles/polymer coating layers (one, two, and three). To achieve one, two, three polymer coating layers, all the steps of the single-coating cycle (Fig. 1) were repeated for the times corresponding to the coating indicated layers. The results of the three variables tested were elaborated following the design of experiments (DOE) based on a three-factor factorial design: a) polymer solution concentration; b) number of coating cycles (polymer coating layers), and c) soaking time. Each test was performed in triplicate. The results in terms of polymer coating efficiency and scaffold porosity were evaluated with analysis of variance P value of <0.05 (Fig. 2).

In addition to the parameters evaluated through DOE, morphological characterization together with cell response evaluation was performed on the scaffolds. The polymer coating film should not occlude or decrease in a significant manner scaffold porosity that should be as close as possible to the original porosity of noncoated scaffold (80%), with interconnected pores whose size should range between 250 and 500 μm to promote cell proliferation and suitable exchanges of nutrients and discharge of cell metabolites. The polymer film should positively affect the *in vitro* cell attachment and proliferation.

Scaffold Characterization

Polymer Coating Efficiency Evaluation. Polymer coating efficiency (CE) was quantitatively measured by weight difference between the dry-coated Orthoss® block (W_1) and the Orthoss® block before coating (W_0) divided by the weight of the dry Orthoss® blocks before coating, according to Eq. (1). The resulting value represents the milligrams of PLGA per milligram of Orthoss® block and permits the direct comparison among different scaffolds because it is independent from the scaffold weights.

$$CE = \frac{W_1 - W_0}{W_0}; \quad (1)$$

Porosity Evaluation. The apparent density and porosity values of the Orthoss® blocks and PLGA-coated Orthoss® blocks were measured using a modified liquid displacement method (21) with ethanol as the displacement liquid.

A weighted polymer scaffold (W) was immersed in a graduated cylinder containing a known volume (V_1) of ethanol. The sample was kept in the nonsolvent for 10 min, and then a set of evacuation-pressurization cycles was conducted to force the ethanol into the pore structure. Cycling was continued until no air bubbles were observed leaving the scaffold surface. The total volume of the ethanol and ethanol-soaked scaffold was then recorded as V_2. The volume difference ($V_2 - V_1$), represented the volume of the scaffold skeleton. The ethanol-soaked scaffold was then removed from the cylinder and the residual ethanol volume was recorded as V_3. The volume ($V_1 - V_3$), that is the ethanol volume retained in the porous scaffold, was defined as the scaffold pore volume. The total scaffold volume was calculated as follows:

$$V = (V_2 - V_1) + (V_1 - V_3) = V_2 - V_3$$

The apparent density of the scaffold (d) was expressed as:

$$d = W/(V_2 - V_3)$$

In addition, the porosity of the scaffold (ε) expressed as percentage (in percent) was calculated by:

$$\varepsilon(\%) = (V_1 - V_3)/(V_2 - V_3)*100$$

Scanning Electron Microscopy. Scanning electron microscopy (SEM) analyses were performed with the aim of analyzing the morphology of Orthoss® block structure after coating. The analysis can give information about: a) if and how the coating process led to modify scaffold morphology in terms of trabecular structure collapse and pores clogging; b) the spreading of polymer coating on the outer and inner scaffold surfaces.

SEM analyses on Orthoss® blocks and PLGA coated Orthoss® blocks were performed by a Zeiss EVOMA10 electron microscope (Carl Zeiss Oberkochem). Samples kwere gold-sputtered and high vacuum analysed. To evaluate the internal morphology, scaffolds were included into an agarose matrix (3%, *w*/*v*) and then sliced using a cryotome (Cryostat, Leica CM1850) to obtain slides of the scaffold showing the internal matrix.

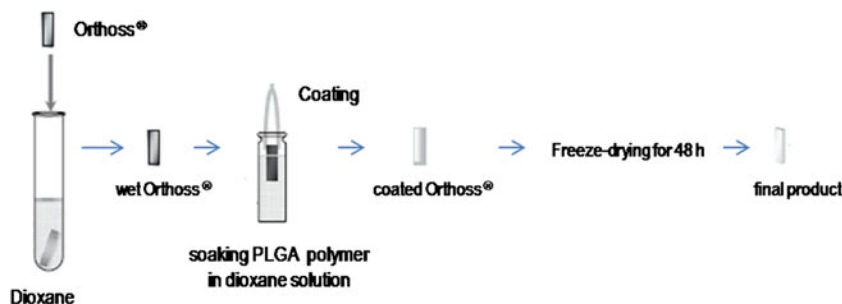

Fig. 1. Scheme of a single cycle of the Orthoss® set up coating process

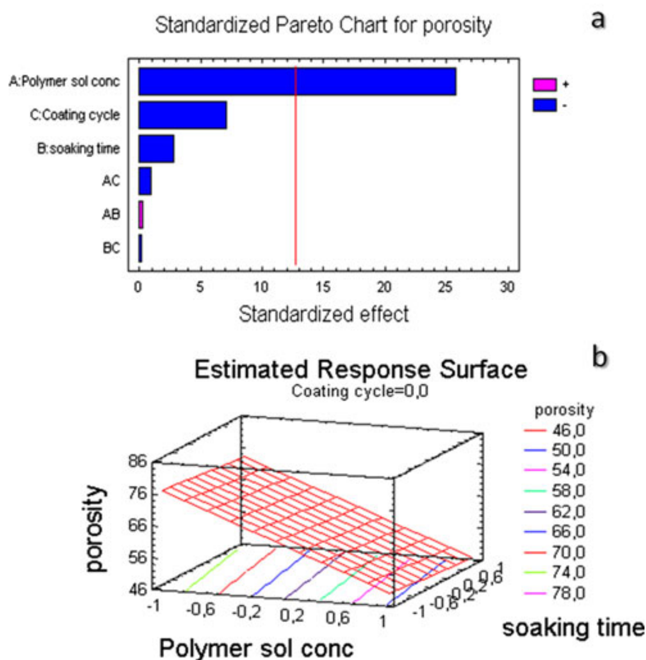

Fig. 2. DOE elaboration of process variables: **a** standardized Pareto chart and **b** results of surface response

Cytotoxicity Studies. The effects of PLGA polymer coating on cell viability were assessed with the 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium (MTT) assay, using six-well cell culture cluster with 400 K_{cells} (fibroblasts) primary cultures plated in contact to the PLGA coated Orthoss® blocks surface.

Briefly, the fibroblasts were cultured in wells in DMEM supplemented with FBS for 24 h at 37°C, then the media were removed and fresh DMEM (without serum) with the samples (Orthoss® blocks) was added. Controls were represented by fibroblasts cultured in wells in DMEM supplemented with FBS for 24 h at 37°C, then the media were removed and fresh DMEM (without serum) was added. After 48 h, 25 μL of MTT solution (5 mg/mL in DMEM) were added into the wells. Cells were incubated for 2 h at 37°C to allow MTT reduction by mitochondrial dehydrogenase in viable cells. After 2 h, Orthoss® samples were removed from the wells, and a suitable detergent was added to dissolve the resulting blue formazane crystals. The commonly used MTT protocol to dissolve formazane crystals was modified to avoid interference of scaffold material with DMSO: THF was used to dissolve the formazane crystals of cells adhered and entrapped to the coated Orthoss blocks, while DMSO was added to the well to dissolve the formazane crystals of cells adhered to bottom well. Results were revealed by a multiwell scanning spectrophotometer (Microplate Reader Model 680, Bio-Rad Laboratories, USA). The optical density (OD) was measured at 595 nm (solutions obtained in THF) and at 570 nm (solutions obtained in DMSO, always with 655 nm as reference wavelength. Cell viability was calculated as the percentage of untreated cells (control).

The goal of the test was to evaluate the more suitable coating conditions in terms of biological response of cultured cells. For this reason it was performed on the PLGA Orthoss® blocks coated with: (1) PLGA solutions of increasing concentrations (0, 4, 6, 7.2, and 10% (*w*/*v*)) and increasing times of soaking into the coating polymer solution (0, 30, 60, and 90 min), (ii) 4% (*w*/*v*) polymer solution concentration, 30 min soaking time, and increasing number of polymer layers (0, 1, 2, and 3). The samples numbered as (0) correspond to noncoated Orthoss® block tested as reference sample. Cells without Orthoss® block were seeded and tested as control. Cell viability is expressed as Viability% and calculated as percentage of cells seeded on the plastic well bottom (control).

In Vitro Proliferation Study. The biologic test was performed to study the effects of scaffold PLGA coating on long term cell proliferation. The studies were assessed on Orthoss® blocks and on Orthoss® blocks PLGA coated scaffolds using six-well cell culture cluster. All samples were sanitized by embedding them three times into 50 mL of ethanol (70%, *w*/*v*) and then washing them in 50 mL of sterile physiological solution (0.9%, *w*/*v*) prior to cell culture. Fibroblasts primary cultures were seeded on the sanitized samples at a concentration of 10,000 cells/mg of polymer corresponding to 400 K cells/scaffold. The samples were kept at 37°C in an atmosphere of 5% CO_2, for incubation periods of 21 days, the medium was changed by fresh medium every 2 days. At scheduled times (7, 10, 14, and 21 days) scaffolds were removed from their respective wells and placed in new wells, after each time point, to ensure that only cells attached to the test samples were considered for analysis. Control cultures were grown on the bottom of wells with the same protocol, uncoated Orthoss® blocks samples were processed and tested as references. Cell proliferation was determined with the MTT assay. At scheduled times, MTT working solution was added into the wells and the MTT test was carried out as explained in

Table I. Coating Efficiency and Porosity of PLGA-Coated Orthoss® Block Expressed as a Function of Coating Polymer Solution Concentration and Soaking Time, for a Single-Coating Cycle

Polymer solution concentration (%, *w*/*v*)	0	4			6			7			10		
Soaking time (min)	–	30	60	90	30	60	90	30	60	90	30	60	90
Coating efficiency	–	0.2	0.21	0.21	0.21	0.22	0.23	0.19	0.18	0.18	0.17	0.18	0.19
Porosity (%)	80	79.5	79.3	77.5	65.2	63.3	61.3	67.4	65.3	60.2	54.3	53.0	50.7

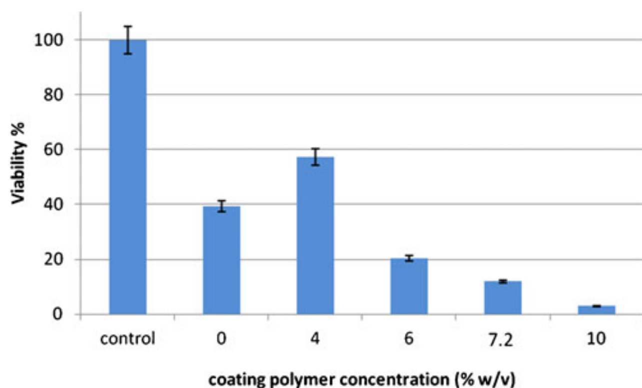

Fig. 3. Effect of coating polymer concentrations on fibroblast cells seeding (single-coating layer and soaking time, 30 min)

the previous paragraph. The OD was measured at 595 and 570 nm with 655 nm as reference wavelength, as explained in the previous paragraph. Results are expressed as number of cells determined at the scheduled times and calculated in reference to a calibration curve set up for fixed cells numbers/concentrations

Confocal Microscopy. Confocal microscopy was used with the purpose to highlight cell proliferation during incubation on the PLGA-coated scaffolds. For the confocal microscopy studies, the Orthoss® blocks and on the Orthoss® blocks PLGA coated scaffolds incubated for 7, 14, and 21 days respectively, as explained here above for cell proliferation studies, washed with PBS, fixed with 70% ethanol for 10 min and stained with 300 μL of DAPI (1 g/mL) to highlight the cell nucleus. The specimens were examined under an inverted confocal laser scanning microscope (Leica TCS SP2, Leica Instruments, Germany). A triple set of samples was performed for each time point and six images of each sample/time point were analyzed.

Statistical Analysis

Data were expressed as mean±standard deviation. Comparison of mean values was performed using one-way analysis of variance. A statistically significant difference was considered when $P<0.01$.

Table II. Coating Efficiency and Porosity of PLGA Coated Orthoss® Block Expressed as a Function of the Number of Coating Cycles (Polymer Coating Solution Concentration, 4% (*w*/*v*) and Soaking Time for Each Single Coating Cycle, 30 min)

	Number of polymer coating cycles (coating layers)			
	0	1	2	3
Coating efficiency	–	0.2	0.21	0.31
Porosity (%)	80	81.5	62.7	55.0

RESULTS AND DISCUSSION

The goal of the experimental set up protocol was to achieve a continuous and homogeneous polymer coating on the scaffold surface without occluding pores and decreasing scaffold porosity. In these terms, the parameters evaluated, polymer solution concentration, soaking time, and number of polymer coating cycles are interconnected. The standardized Pareto chart originated from DOE elaboration shows (Fig. 2a) that polymer solution concentration is the only parameter that significantly affects the process. The 3D plot of the estimated response surface (Fig. 2b) shows that scaffold porosity is dependent on polymer solution concentration and independent from soaking time. Starting from DOE elaboration, the results are reported in Table I in terms of coating efficiency obtained from the ratio between the weights of PLGA coating and Orthoss® block. Coating efficiency permits the direct comparison among different scaffolds because the parameter is independent from the scaffold weight. Moreover, its comparison with scaffold porosity gives the information about how polymer film spreads on the scaffold. In the same Table I the results in terms of porosity evaluated at increasing polymer solution concentrations and soaking times are reported. The shortest soaking time in the polymer solution was 30 min because preliminary experiments (data not shown) demonstrated that it takes at least 20 min. to soak the Orthoss® block with the 1,4-dioxane polymer solvent. Results show that coating efficiency does not vary significantly increasing polymer solution concentration up to 6% (*w*/*v*), while it decreases for higher polymer solution concentrations. These results (Table I) can be explained by the high polymer solutions

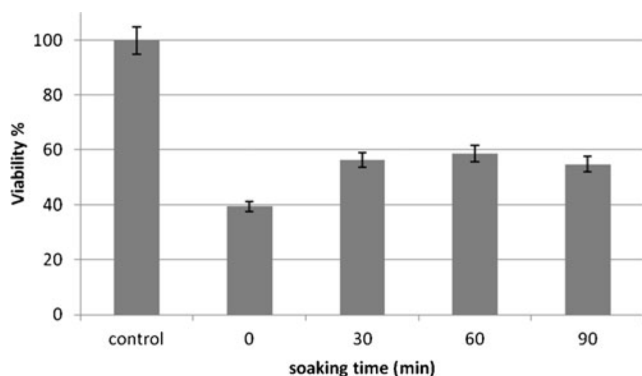

Fig. 4. Effect of soaking time on fibroblast cells seeding (single-coating layer and polymer solution concentration, 4% (*w*/*v*))

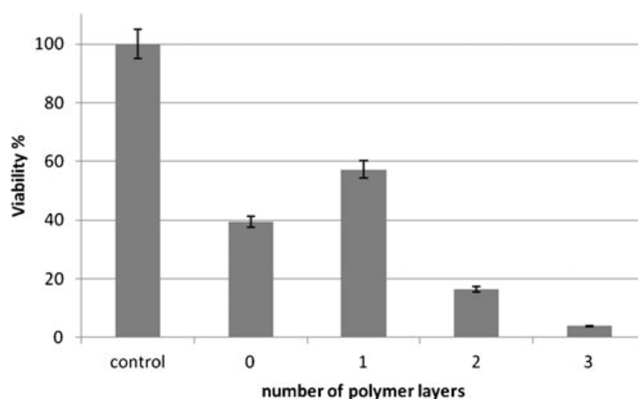

Fig. 5. Effect of polymer layer number on fibroblast cells seeding (polymer solution concentration, 4% (*w*/*v*) and soaking time, 30 min)

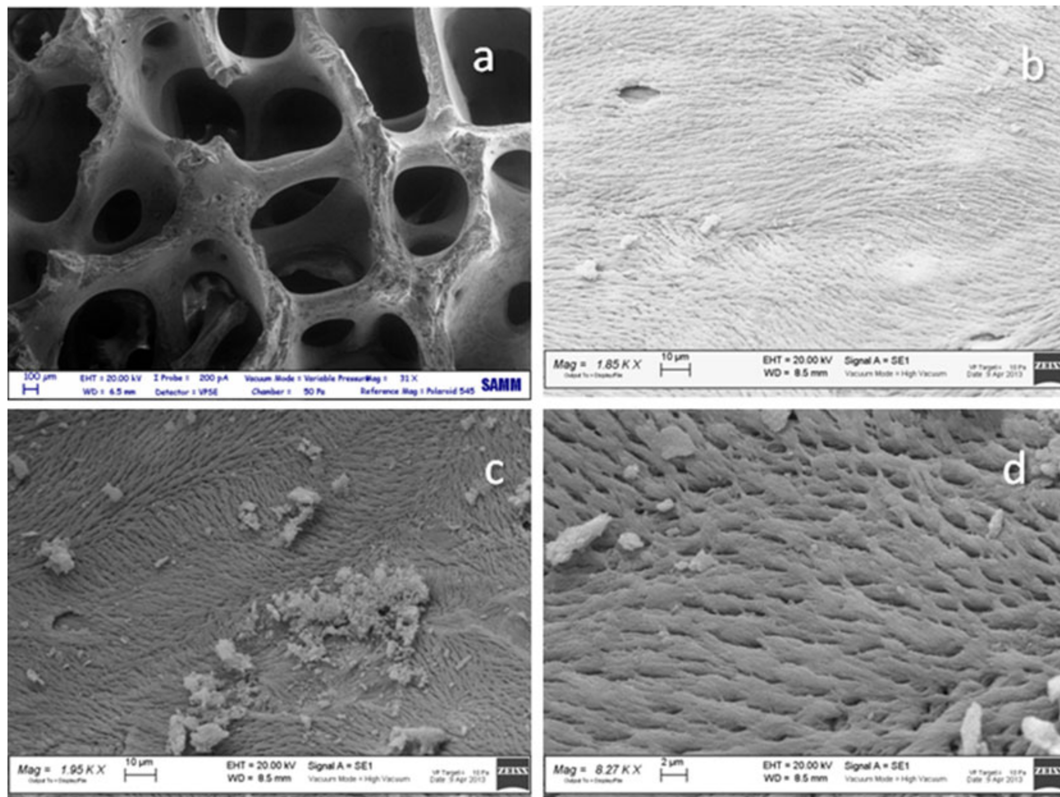

Fig. 6. SEM images of PLGA-coated Orthoss® block: **a** trabecular pattern and porosity at ×31 magnifications; **b** outer surface at ×1.85 K magnifications **c** inner surface at ×1.85 K magnifications, and **d** inner surface at ×8.27 K magnifications

viscosities at concentrations higher than 6% that lead the polymer to settle on the scaffold surface with not homogeneous pattern. Nevertheless, scaffold porosity keeps at values of about 80%, comparable to those of uncoated scaffolds, when 4% (*w*/*v*) polymer solution concentration was used, and it decreases sharply for higher polymer solution concentrations. Also these results (Table I) can be explained by the increased polymer solutions viscosities at concentrations higher than 4% that leads the polymer to settle on the scaffold surface clogging Orthoss® block pores. Good-coating-efficiency results were obtained with polymer solution concentrations of either 4% and 6% (*w*/*v*), but the lower polymer solution concentration is able to maintain 80% porosity. This means that at 4% (*w*/*v*) concentration the polymer spreads as an homogenous film on the Orthoss® block surface and it does not clog scaffold pores. Soaking time does not lead to significant changes in coating efficiency for all the polymer concentrations tested, but significant reduction of porosity values is highlighted for 90 min soaking time. The results of cell viability test confirmed the hypothesis suggested by the porosity and coating efficiency results. To highlight these findings, Figs. 3 and 4 report the results in terms of cell viability obtained on Orthoss® blocks with different coating polymer concentrations (4, 6, 7.2, and 10% (*w*/*v*)) and 30 min soaking time, while in Fig. 4 are plotted the results of cells viability as a function of soaking time for 4% (*w*/*v*) polymer solution concentration. As explained in the experimental sections, these results are reported as percentages with respect to controls. Orthoss® blocks coated with 4% (*w*/*v*) polymer concentration show an improvement of about 30% in cell seeding ability with respect to not coated Orthoss® blocks, while Orthoss® blocks coating with higher polymer solution concentrations leads to a drastic and significant reduction of cell viability. The different soaking times tested (Fig. 4) do not significantly affect the cell seeding capacity, and similar results were obtained for the Orthoss® blocks coated with all the different polymer concentrations tested (data not reported). The positive results highlight that the polymer coating as such, and in suitable conditions (4% polymer solution concentration, 30 min soaking time) stimulates cell seeding. The finding is encouraging future possible development of the polymeric coating as growth factors reservoir. The scaffold porosity reduction is the reason of the reduced cell seeding capacity

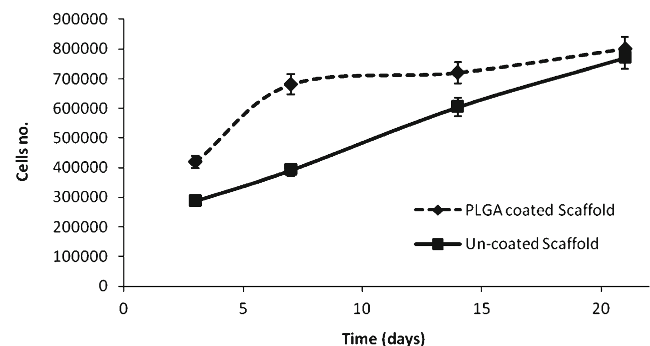

Fig. 7. Results of cells proliferation study (polymer solution concentration, 4% (*w*/*v*), soaking time, 30 min, and one-coating cycle)

observed for coatings with polymer solution concentrations higher than 4%. In fact, scaffold porosity is of outmost importance because it creates an environment suitable cell adhesion and proliferation. Macroporosity ranging between 250 and 500 μm is needed to allow cells to penetrate inside the scaffold, adhering to it and proliferating in the 3D structure; microporosity ranging between 50 and 150 μm is important to permit the exchange of nutrients and elimination of cell metabolites and discharge products. For this reason porosity reduction leads to reduced cell seeding capacity. Starting from these results, 4% (*w*/*v*) polymer solution concentration and 30 min soaking time were selected to submit the Orthoss® blocks to more than one-coating cycle. The results reported in Table II show that indeed a significant porosity decrease is highlighted for Orthoss® blocks submitted to two-coating cycles. Moreover, the decrease in porosity does not correspond to an increase in coating efficiency, leading to the conclusion of a not homogeneous polymer casting on the scaffold surface. The data are consisting with the results of biologic test reported in Fig. 5, showing a cell viability absolute value of about 18% and about 5% for 2 and 3 cycles coating respectively, corresponding to a cell viability reduction of 42% and 55%, with respect to the results cell viability referred to the Orthoss® blocks submitted to a single coating cycle. The results confirm that scaffold porosity, as known from the literature (2,4,21), and as explained here above, plays an essential role in cell proliferation. Reduced scaffold macro or microporosity can lead to cell necrosis.

As shown in Fig. 6a, SEM analyses highlighted that the coating process performed in all the conditions tested did not lead to collapse the scaffold trabecular structure that is fundamental to permit cell adhesion and proliferation. Moreover, the scaffold macroporosity ranging about 300–500 μm and the pores interconnectivity is clearly shown. SEM analysis at higher magnifications (Fig. 6b–d) permits to highlight the homogeneous spreading of polymeric coating onto both the outer and inner scaffolds surfaces, and the regular weave.

The results of long term cell proliferation study performed on the selected coated Orthoss® blocks (4% polymer solution, one-coating cycle and 30 min soaking time), are reported in Fig. 7. They show that, in the first 15 days of cell incubation, coated Orthoss® blocks significantly improve cell proliferation with respect to the uncoated ones. Only after this time that the uncoated Orthoss® blocks behave as the coated ones. These data are positive and valuable because they demonstrate that the coated Orthoss® block scaffold are faster colonized by cells, thus they could be more rapidly integrated in an *in vivo* environment. Moreover, the results confirm that polymer coating is not cytotoxic and did not release any leachable solvent residuals or polymer degradation products formed during the incubation time that could be hazardous for cells. Indeed the positive results encourage future possible

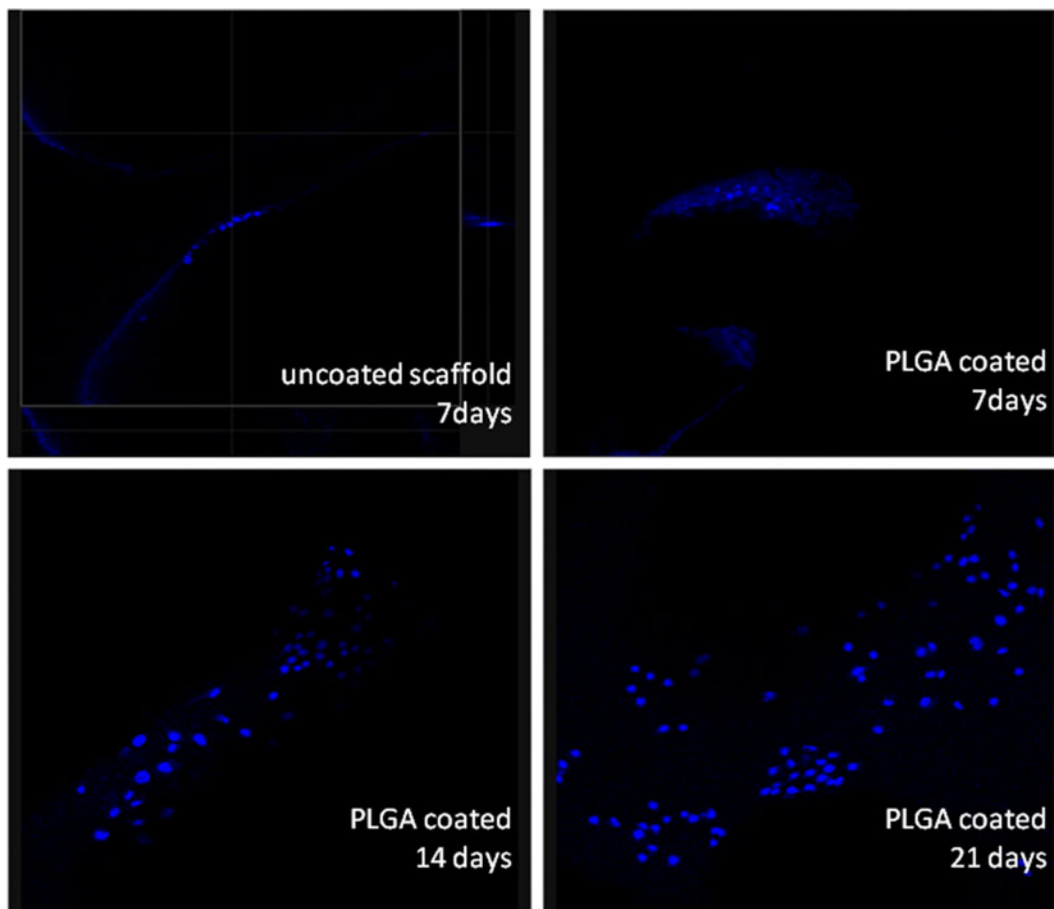

Fig. 8. Confocal microscopy images of fibroblasts cell proliferation on PLGA-coated scaffolds at different incubation times. Cell proliferation on the uncoated scaffolds at 7 days is reported as reference

development of the polymeric coating as growth factors reservoir.

Confocal microscopy analyses performed on the Orthoss® blocks and on the Orthoss® blocks PLGA coated scaffolds incubated for 7, 14, and 21 days respectively, qualitatively demonstrate the satisfactory cell colonization onto Orthoss® blocks PLGA coated scaffolds surfaces progressively increasing with time. As shown in the pictures taken at 7, 14, and 21 days, living cells are homogeneously spreading and proliferating on the Orthoss® blocks PLGA coated scaffolds (Fig. 8). Cell colonization onto uncoated Orthoss® blocks as been reported only at day 7, as control.

CONCLUSIONS

The following conclusions can be drawn from the results obtained.

The designed polymer coating protocol did not affect the trabecular structure of the original Orthoss® block and it is suitable to prepare PLGA-coated Orthoss® block.

The polymer solution concentration and the number of polymeric layers are the main variables affecting the coating efficiency and the porosity of the PLGA-coated Orthoss® block. Four percent PLGA solution concentration, 30 min soaking time resulted to be the most suitable conditions to achieve good polymer coating efficiency maintaining the starting scaffold porosity thus keeping suitable conditions for cell adhesion and proliferation.

The obtained positive results in terms of homogenous coating on the Orthoss® block surface, without clogging scaffold pores, represent the first step for further investigation involving loading of growth factors into the polymeric coating. In these terms, the growth factor loaded polymer coating could act as a modified release drug delivery system at the site of insertion, further promoting tissue regeneration. Nevertheless, the preliminary investigation performed was important to set up the suitable Orthoss® block-coating protocol.

Moreover, the obtained results show how PLGA coating Orthoss® blocks improves the capacity of the scaffold to stimulate the growth and proliferation of fibroblasts cells. This strategy can be useful to achieve a medical device with improved performances.

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