

Gelatine/PLLA sponge-like scaffolds: morphological and biological characterization

Luigi Lazzeri · Maria Grazia Cascone ·
Serena Danti · Lorenzo Pio Serino ·
Stefania Moscato · Nunzia Bernardini

Received: 4 October 2005 / Accepted: 4 January 2006 / Published online: 3 February 2007
© Springer Science+Business Media, LLC 2007

Abstract Biodegradable synthetic polymers such as poly(lactic acid) (PLA) are widely used to prepare scaffolds for cell transplantation and tissue growth, using different techniques set up for the purpose. However the poor hydrophilicity of these polymers represents the main limitation to their use as scaffolds because it causes a low affinity for the cells. An effective way to solve this problem could be represented by the addition of biopolymers that are in general highly hydrophilic. The present work concerns porous biodegradable sponge-like systems based on poly(L-lactic acid) (PLLA) and gelatine. Morphology and porosity characteristics of the sponges were studied by scanning electron microscopy and mercury intrusion porosimetry respectively. Blood compatibility was investigated by bovine plasma fibrinogen (BPF) adsorption test and platelet adhesion test (PAT). The cell culture method was used in order to evaluate the ability of the matrices to work as scaffolds for tissue regeneration. The obtained results indicate that the sponges have interesting porous characteristics, good blood compatibility and above all good ability to

support cell adhesion and growth. In fact viable and metabolically active animal cells were found inside the sponges after 8 weeks in culture. On this basis the systems produced seem to be good candidates as scaffolds for tissue regeneration.

Introduction

Tissue regeneration by cell transplantation requires the development of substrate materials able to provide microenvironments for cell–matrix interactions by mimicking biological conditions.

Several basic properties [1] are required to a material to be used as scaffold for cell transplantation and tissue growth, such as biocompatibility, mechanical stability to support and transfer loads, sterilizability [2] and biodegradability. This last feature is extremely important because a biodegradable material allows the penetration and growth of cells into the construct while eliminating the need for a second surgery to remove the implant [3–5].

In addition, a scaffold must be highly porous to provide adequate space for cell seeding, growth and extracellular matrix production, and must have a uniformly distributed and interconnected pore structure, so that the cells are easily distributed throughout the device and an organized network of tissue constituents can be formed [6–8].

Synthetic biodegradable polymers such as poly(lactic acid) (PLA) are widely used for the preparation of porous scaffolds by different techniques set up for the purpose [9–12]. The main limitation to the use of PLA-based systems is represented by their low hydrophilicity

L. Lazzeri · M. G. Cascone · S. Danti (✉) ·
L. P. Serino
Department of Chemical Engineering, University of Pisa,
Via Diotisalvi 2, 56126 Pisa, Italy
e-mail: s.danti@ing.unipi.it

S. Moscato · N. Bernardini
Department of Human Morphology and Applied Biology,
University of Pisa, Via Roma 55, 56126 Pisa, Italy

Present Address:

S. Danti
Department of Oncology and Transplantations, University
of Pisa, Via Roma 55, 56126 Pisa, Italy

that causes a low affinity for the cells. Unlike synthetic polymers, biological polymers are in general highly hydrophilic although their mechanical properties are poor [1, 13]. Therefore scaffolds based on pure biopolymers present a good affinity for the cells but their mechanical stability results inadequate. In this respect the addition of a biopolymer to a synthetic component such as PLA represents an interesting way to produce a “bioactive scaffold” that can be considered as a system showing at the same time adequate mechanical stability and high cell affinity.

The aim of the present work was the preparation of porous sponge-like systems based on PLA and gelatine and their evaluation as scaffolds for tissue regeneration. The study was mainly focused on the evaluation of cellular response of a relatively short time (until 8 weeks) in which adhesion and early proliferation processes occur. It is worthwhile to underline that in the investigated time period biodegradation of PLLA matrices can be neglected. The technique selected for the preparation of the matrices is based on a simple water-in-oil (W/O) emulsion [14]. Gelatine [15] was selected as biological component not only because of its well known ability to stimulate cell adhesion, but also because its ability to foam by stirring allows the preparation of a highly porous matrix eliminating the need for the addition of surfactants that could compromise the biocompatibility properties of the final material.

Morphology and porosity characteristics of the produced matrices were studied by scanning electron microscopy and mercury intrusion porosimetry respectively.

Blood compatibility was investigated by two different in vitro tests: bovine plasma fibrinogen (BPF) adsorption test and platelet adhesion test (PAT).

The cell culture method was used in order to evaluate the ability of the matrices to work as scaffolds for tissue regeneration.

Materials and methods

Matrices production

Three gelatine (gelatine type B from bovine skin, 75 bloom, Sigma-Aldrich, Milan, I) solutions with concentration of 0.25, 0.5, and 3% (w/v) were prepared in distilled water. Each solution was added drop by drop to a 2.5% solution of poly(L-lactic acid) (PLLA) (average molecular weight MW 380,000, Boehringer, Ingelheim, D) in chloroform under stirring at

10,500 rev min⁻¹ for 5 min. A foam was obtained that was immediately frozen in liquid nitrogen and then freeze-dried producing a sponge-like matrix. Depending on the initial gelatine concentration (0.25, 0.5, and 3%) matrices with the following Gelatine/PLLA composition ratios were obtained: 9/91, 17/83, and 55/45 (w/w).

Scanning electron microscopy

The internal structure of the produced sponges was examined by a scanning electron microscope (SEM) Jeol JSM-5600 LV. The samples were freeze-dried and then mounted on aluminum stubs and coated with gold prior to examination.

Mercury intrusion porosimetry

Hg intrusion porosimetry was used to evaluate the porosity characteristics of the sponges.

Pore size in the range 0.007–120 μm in diameter was evaluated by Hg intrusion using a Carlo Erba Pascal 140 Porosimeter, equipped with an automatic recording of intruded Hg volume. The pore volume distribution was obtained from the derivative curve of the cumulative intruded pore volume as function of pore diameter. This latter parameter is related to the measured pressure according to the Washburn model equation [16], developed for the intrusion of a cylindrical shape pore:

$$d = (4\gamma\cos\theta/P) \cdot 10$$

The cylindrical diameter d (μm) of the filled pores is inversely proportional to the intrusion pressure P (kg cm⁻²), when Hg surface tension γ (0.48 N m⁻¹) and the contact angle θ between Hg and the material are constant. Values of the contact angle around 140°, were measured for the matrices with different composition.

Blood compatibility tests

In order to evaluate the compatibility of the produced sponges with blood, two different in vitro tests were performed: the BPF adsorption test [17] and the PAT [18]. The first measures the absorption of fibrinogen on the surface of the sponges while the second evaluates the ability of the sponges to support platelet adhesion.

The BPF absorption test was performed in duplicate, using a slight modification of the procedure described by Ishihara et al. [17].

Briefly the BPF (MW 34,000, Sigma) was dissolved in phosphate buffer saline (PBS) with concentration of 1 mg mL^{-1} . Sponge-like samples were immersed in the solution at 37°C under mild shaking. To consider the adsorption of BPF on the wall of the vials containing the samples, a blank test was performed. After a series of immersion times (10, 20, 40, and 60 min), samples were removed, and the changes of the concentration in the solution were investigated using an UV-spectrophotometer at $\lambda = 280 \text{ nm}$.

The PAT was performed in quintuplicate, in accordance with the procedure reported by Han et al. [18]. After samples were equilibrated with PBS overnight, they were immersed in platelet rich human plasma and incubated at 37°C with mild shaking. After 19 h the samples were taken out from the solution and rinsed five times with PBS in order to remove the weakly adsorbed platelets. Then the strongly adsorbed platelets were fixed on the sponge surfaces by immersing the samples in 2% (v/v) glutaraldehyde aqueous solution at room temperature for 2 h. After that fixation the sponges were dried in atmosphere for 48 h and under vacuum for 5 h. The dried samples were coated with gold and analysed by scanning electron microscopy.

A slide treated with fibrinogen, protein that favours platelet adhesion, was used as a positive control.

Biological characterization

Fibroblasts were isolated from ovine embryonic lung explants and cultured in 80 cm^2 flasks in a complete medium composed of culture media D-MEM with 10% foetal bovine serum and 1% L-glutamine–penicilline–streptomycin (Sigma-Aldrich, Milan Italy). After sterilization with ethanol, Gelatine/PLLA sponges with composition 9/91 were placed on the bottom of 24-well TCPS culture dishes. Fibroblasts were seeded onto the materials at a seeding density of 5.7×10^4 cells/sample.

The presence of viable cells into the matrices was qualitatively evaluated by the use of 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT) assay: 8 weeks after seeding, culture medium was removed and replaced with MTT solution. After 4 h incubation the solution was removed and the matrices were observed by optical microscope.

After 8 weeks in culture some samples were processed for morphological, histochemical and immunocytochemical analysis.

The constructs were washed in PBS and fixed in 4% phosphate-buffered paraformaldehyde overnight at 4°C . Sponges were then dehydrated through a graded series of ethanol, cleared with xylene and embedded in

paraffin according to standard histological techniques. Sections ($8 \mu\text{m}$ thick) were stained with haematoxylin and eosin for morphological analysis. Histochemical analysis was carried out by Alcian Blue staining to evaluate glycosaminoglycans production or by periodic acid Schiff (PAS) reaction to detect glycoproteins expression and localization. Immunocytochemistry was performed to evaluate elastin, collagen I and fibronectin expression. Rabbit polyclonal antibodies (Sigma Chemical Co, St. Louis, MO, USA) to elastin, collagen I and fibronectin were used as the primary antibodies diluted in 0.1% BSA/1 × PBS and incubated overnight at 4°C . The detection of antigenic sites was carried out using LSAB+ kit (Dakopatts, Milan, Italy) as previously reported [19]. The reaction was developed by incubating samples in the substrate-chromogen solution (1 mg mL^{-1} 3,3'-diaminobenzidine tetrahydrochloride (DAB) containing 0.02% H_2O_2) for 5 min in the dark. Slides were then mounted with Universal Mount and observed with a DMRB Leica microscope. Control experiments were performed incubating the specimens with the omission of primary antibody.

Results and discussion

Scanning electron microscopy was employed to evaluate the morphological characteristics of the materials. The internal structure of the sponges appears porous and no sign of separation between the two components is visible at the magnification used. However, it was observed that the morphological characteristics of the sponges depend on the gelatine content. The structure of the samples containing 9 and 17% of gelatine is irregular with highly interconnected cavities (Fig. 1a, b). The sample containing 55% of gelatine shows large spheroidal cavities poorly interconnected through a number of small holes in the walls (Fig. 1c).

The effect of gelatine content on the porosity of the sponges was also confirmed by mercury intrusion porosimetry, performed to investigate pore size distribution inside the materials.

The results revealed a distribution of pore size in the 10–110 μm range with two main maxima falling within the 10–20 μm and 50–100 μm classes, respectively (Fig. 2).

Samples with 9 and 17% of gelatine show almost the same pore distribution. They have a higher connectivity between pores with respect to the sample containing 55% of gelatine, as revealed by the higher value of the distribution curve in the 10–20 μm class.

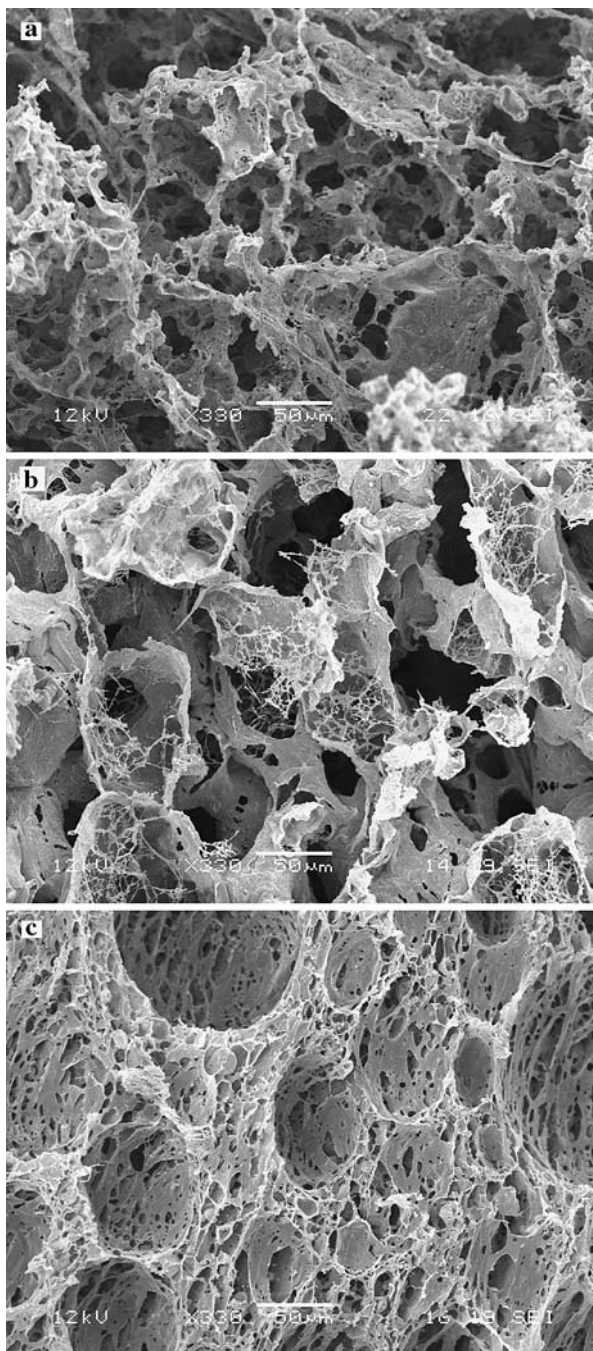


Fig. 1 SEM images of different Gelatine/poly(L-lactic acid) (PLLA) sponges with compositions (w/w): 9/91 (a), 17/83 (b) and 55/45 (c)

As expected, the sample containing 55% of gelatine has a greater pore volume in the range 50–100 μm , related to the large spherical pores, and a smaller fraction of pores in the 10–20 μm range, related to the holes in the walls.

The effect of gelatine on the morphology and pore size distribution of the sponges is reasonably ascribable

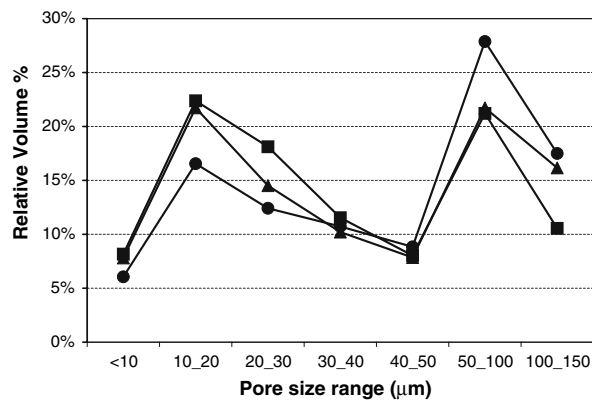


Fig. 2 Pore size distribution expressed as relative volume for sponges with compositions (w/w): 9/91 (▲), 17/83 (■) and 55/45 (●)

to the porogen nature of gelatine: the shape of the pores is in fact more well defined when the gelatine content is higher.

In the subsequent characterization only samples containing 9 and 17% of gelatine were considered because of their porosity characteristics more suitable for cell colonisation.

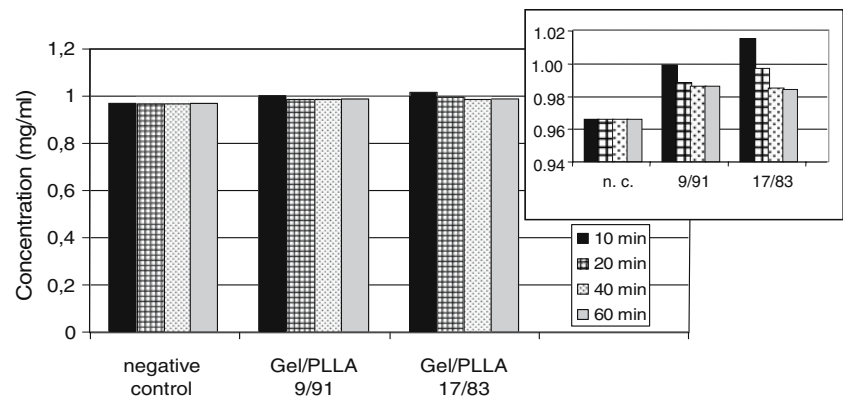
A good compatibility of these sponges with blood was observed. Blood compatibility was investigated by two different in vitro tests based on fibrinogen adsorption and platelet adhesion, respectively.

Fibrinogen is one of the most important globular proteins circulating in the blood. It plays a central role in the regulation of haemostasis and thrombosis by participating in blood coagulation processes and facilitating adhesion and aggregation of platelets.

In the fibrinogen absorption test, sponges containing 9 and 17% of gelatine and glass, as a negative control, were used. Each sponge was immersed for different time periods into the fibrinogen solution. Measuring fibrinogen remained in the solution after the sample was removed allowed the calculation of the amount of fibrinogen adsorbed by the sample. Figure 3 shows the results of the test and reveals that no very significant difference was observed between the tested materials and the control. The negative control shows a very low adsorption of fibrinogen as revealed by the protein concentration decreasing from 1 mg mL^{-1} to 0.97 mg mL^{-1} . In addition, the adsorption of fibrinogen onto the control is complete within the first 10 min.

Both types of sponges tested also show a very slight adsorption, but the adsorption was complete after about 60 min, as better evidenced in the magnified part of Fig. 3. Furthermore, the sponges appear to behave better than the control in the sense that a higher

Fig. 3 Results of bovine plasma fibrinogen (BPF) adsorption test. The concentration of fibrinogen not absorbed by the samples is reported on the ordinate



concentration of protein was found in the remaining solution.

It must be outlined that the results of this test are qualitative and somewhat unreliable. Several reasons can be cited for this. One is related to the observation that in some cases the concentration of fibrinogen in the solution appears higher than the initial value. This is due to the fact that the sponges can release gelatine which absorbs significantly at the same wavelength as fibrinogen and perturbs its spectrophotometric determination. Another important limit is related to the fact that no particular care was taken over keeping equal the surface areas of samples and control. This, of course, makes the absorption kinetics not comparable. Finally, since the test was performed in duplicate only, no standard deviation was reported and the average values of absorption have to be considered carefully.

The PAT was used to evaluate the interaction of human platelets with the surface of the sponges. The test was performed by dipping the sponges and the control into platelet rich human plasma. The results reported in Fig. 4 show the absence of platelets on the surface of the sponges, as compared with the positive control, and confirm the high degree of blood compatibility of the tested materials.

The ability of the sponges to work as scaffolds for tissue regeneration was investigated by *in vitro* tests based on the cell culture method. Fibroblasts isolated from explants of ovine embryonic lung were characterized and seeded onto Gelatine/PLLA = 9/91 sponges and cultured for 8 weeks. Fibroblasts were chosen because it is well known their high replication capacity *in vitro* and therefore they can be considered an effective tool for preliminary studies of cell adhesion and growth onto a scaffold.

The presence of viable cells into the matrices, at different times after seeding, was demonstrated by the use of MTT test. Samples were added with MTT

solution and after 4 h incubation were observed by optical microscope. The presence into the sponges of viable cells was revealed by the violet colour taken on by the cells because of the formation and storing of formazan into the cytoplasm (Fig. 5).

After 8 weeks in culture, sponges were processed for histological analysis. Staining with haematoxylin and eosin showed the presence of cells with a fibroblast-like morphology and a good spreading of the cells into the scaffold and on its surface. Furthermore the cultured fibroblasts were able to produce glycosaminoglycans and glycoproteins as confirmed by the histochemical staining with Alcian Blue and PAS reaction respectively. Both glycosaminoglycans and glycoproteins were observed in intracellular and extracellular spaces (data not shown).

Specific immunoreactions were observed in spreading cells within scaffold pores compared to the immunonegative control (Fig. 6a). A network of immunoreactive elastin was detected in the matrix (Fig. 6b) whilst the cells displayed an intracellular immunoreaction for collagen I antibodies but no extracellular expression of collagen I molecules was revealed (Fig. 6c). As the immunoreaction for fibronectin is concerned, fibroblasts showed an intense immunoreaction both in the cytoplasm and the extracellular spaces (Fig. 6d).

These results indicate that the produced sponges allow the growth and the maintenance of the fibroblast-like morphology of the cells spreading into the materials. Furthermore fibroblasts resulted metabolically active in the production of extracellular molecules such as glycosaminoglycans, glycoproteins, elastin and fibronectin as demonstrated by the cytochemical and immunocytochemical analysis. With regard to collagen I intracellular localization, it is possible to hypothesize that the embryonic lung fibroblasts employed in this study synthesize a lower amount of this kind of

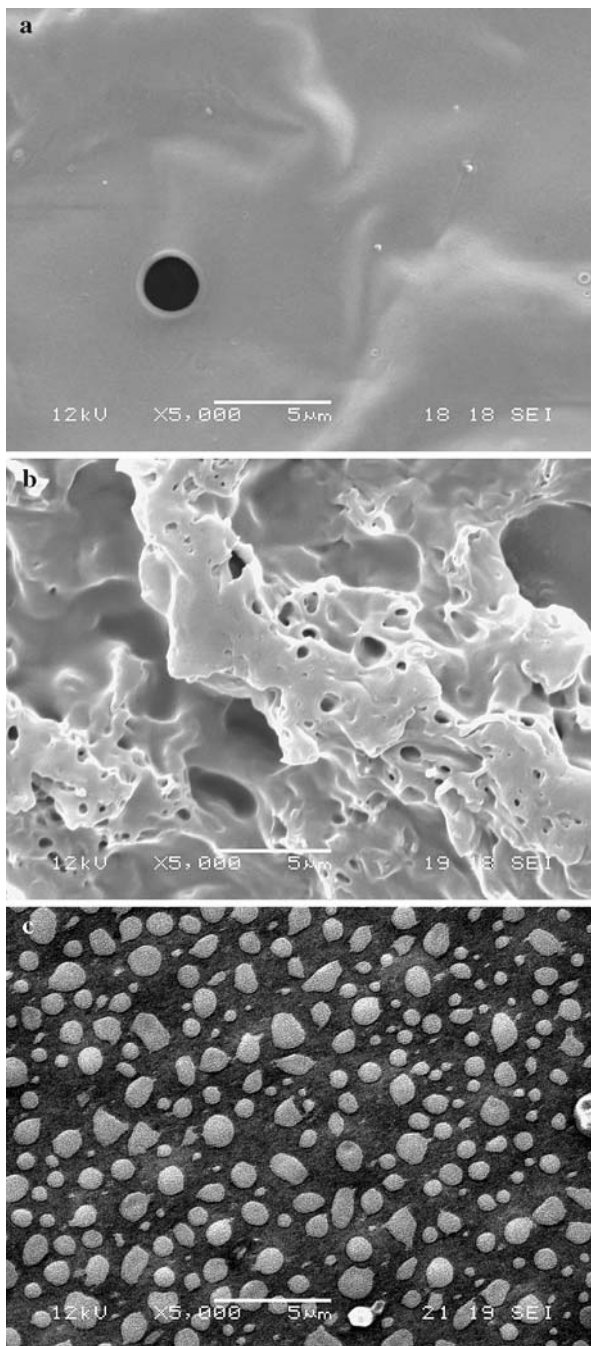


Fig. 4 Results of platelet adhesion test (PAT). No platelets are present on the surfaces of 9/91 (a) and 17/83 (b) Gelatine/poly(L-lactic acid) (PLL) sponges in comparison with the positive control (c)

collagen with respect to the consistent amount of elastin and fibronectin molecules observed in the extracellular spaces. In addition, it is likely that collagen I takes a longer time to be secreted by pulmonary fibroblasts into the matrix in comparison

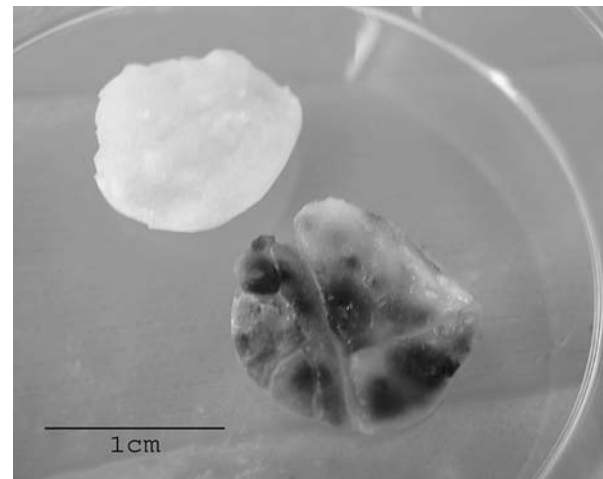


Fig. 5 Images of a Gelatine/poly(L-lactic acid) (PLL) (9/91) sponge before (white) and after (dark) MTT test. The dark colour is due to the presence inside the sponge of a very high number of viable cells (8 weeks after seeding)

with the experimental time at which the morphological analysis was carried out.

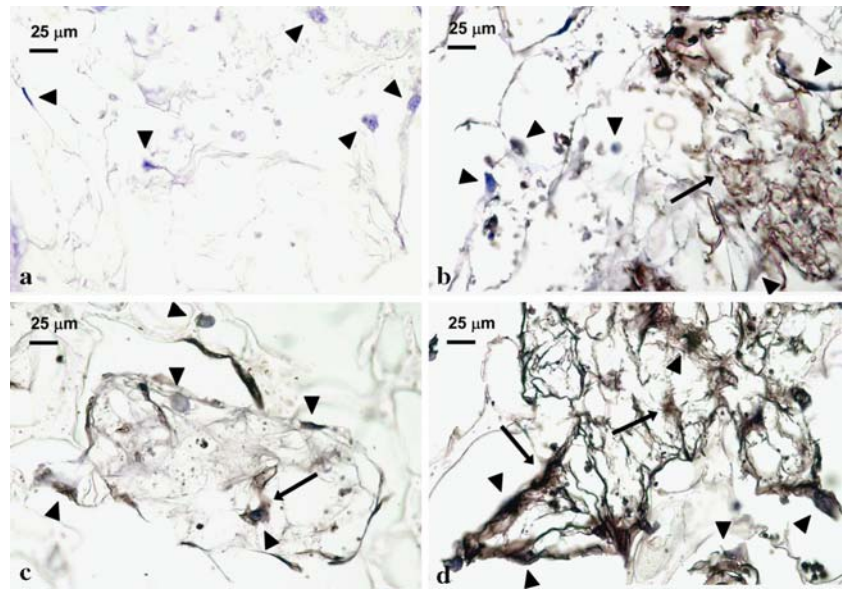
Conclusions

Porous biodegradable sponge-like systems based on PLLA were prepared and tested as scaffolds for tissue regeneration. It was observed that the addition of a biological component, such as gelatine, to the formulation of these systems allowed several advantages to be obtained besides the improvement of the hydrophilicity properties of the materials.

A porous structure can be obtained because of the ability of gelatine to form a foam and therefore a sponge by lyophilization. In addition gelatine is able to affect the porosity characteristics of the produces systems depending on its content. Furthermore it can be supposed that gelatine creates a favourable microenvironment for cell adhesion and growth. The results of in vitro tests based on the cell culture method confirmed the high affinity of the sponges for the cells. In fact viable and metabolically active animal cells were found inside the sponges after 8 weeks in culture.

On the basis of the obtained results it can be concluded that the produced systems seem to be good candidates as tissue engineering scaffolds. Further investigations need to be performed regarding the mechanical properties and the evaluation of the sponges as substrates for human cell culture.

Fig. 6 Immunocytochemical analysis of ovine embryonic fibroblasts grown on a Gelatine/poly(L-lactic acid) (PLLA) = 9/91 sponge. Arrowheads indicate cell nuclei. In (a) negative control obtained by substituting the antibodies with 0.1% BSA/1 × PBS. Elastin was immunodetected mainly at the extracellular level (arrow) (b) Collagen I was expressed within the cytoplasm of the fibroblasts (arrow) (c) Fibronectin was localized both at intracellular and extracellular level (arrows) (d)



Acknowledgments Authors wish to thank Dr. Enzo Sparvoli for porosimetric analysis and Mr. Piero Narducci for SEM analysis.

References

1. B. L. SEAL, T. C. OTERO and A. PANITCH, *Mater. Sci. Eng. R.* **34** (2001) 147
2. J.-P. NUUTINEN, C. CLERC, T. VIRTA and P. TÖRMÄLÄ, *J. Biomater. Sci. Polym. Ed.* **13** (2002) 1325
3. W. H. WONG and D. J. MOONEY, in *Synthetic Biodegradable Polymer Scaffolds* (Birkhausen, Boston, 1997), p. 51
4. J. KOHN and R. LANGER, in *Biomaterials Science: An Introduction to Materials in Medicine*, edited by B. D. Ratner, A. S. Hoffman, F. J. Schoen and J. E. Lemons (Academic Press, New York, 1996), p. 6472
5. M. KELLOMÄKI, H. NIIRANEN, K. PUUMANEN, N. ASHAMMAKHI, T. WARIS and P. TÖRMÄLÄ, *Biomater.* **21** (2000) 2495
6. C. E. HOLY, M. S. SHOICHET and J. E. DAVIES, United States Patent 6,472,210 (2002)
7. F. J. HUA, T. G. PARK and D. S. LEE, *Polymer* **44** (2003) 1911
8. H. NAKAO, S. H. HYON, S. TSUTSUMI, T. MATSUMOTO and J. TAKAHASHI, *J. Dent. Mater.* **22** (2003) 262
9. A. G. MIKOS, A. J. THORSEN, L. A. CZERWONKA, Y. BAO and R. LANGER, *Polymer* **35** (1994) 1068
10. H. LO, M. S. PONTICIELLO and D. K. W. LEONG, *Tissue Eng.* **1** (1995) 15
11. D. J. MOONEY, D. F. BALDWIN, N. P. SUH, J. P. VACANTI and R. LANGER, *Biomater.* **17** (1996) 1417
12. L. D. HARRIS, B. KIM and D. J. MOONEY, *J. Biomed. Mater. Res.* **42** (1998) 396
13. Y. S. CHOI, S. B. LEE, S. R. HONG, Y. M. LEE, K. W. SONG and M. H. PARK, *J. Mater. Sci. Mater. Med.* **12** (2001) 67
14. K. WHANG, C. H. THOMAS, K. E. HEALY and G. NUBER, *Polymer* **36** (1995) 837
15. B. R. YOUNG, W. G. PITT and S. L. COOPER, *J. Coll. Inter. Sci.* **124** (1988) 28
16. E. W. WASHBURN, *Proc. Nat. Acad. Sci.* **7** (1921) 115
17. K. ISHIHARA, A. FUJIIKE, Y. IWASAKI, K. KURITA and N. NAKABAYASHI, *J. Polym. Sci. Polym. Chem.* **34** (1996) 199
18. D. K. HAN, S. Y. JEONG and Y. H. KIM, *J. Biomed. Mater. Res. Appl. Biomater.* **23**(A-2) (1989) 211
19. N. BERNARDINI, F. BIANCHI and A. DOLFI, *Nephron* **81** (1999) 289