

Video Article

A Facile and Eco-friendly Route to Fabricate Poly(Lactic Acid) Scaffolds with Graded Pore Size

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Abstract

Over the recent years, functionally graded scaffolds (FGS) gained a crucial role for manufacturing of devices for tissue engineering. The importance of this new field of biomaterials research is due to the necessity to develop implants capable of mimicking the complex functionality of the various tissues, including a continuous change from one structure or composition to another. In this latter context, one topic of main interest concerns the design of appropriate scaffolds for bone-cartilage interface tissue. In this study, three-layered scaffolds with graded pore size were achieved by melt mixing poly(lactic acid) (PLA), sodium chloride (NaCl) and polyethylene glycol (PEG). Pore size distributions were controlled by NaCl granulometry and PEG solvation. Scaffolds were characterized from a morphological and mechanical point of view. A correlation between the preparation method, the pore architecture and compressive mechanical behavior was found. The interface adhesion strength was quantitatively evaluated by using a custom-designed interfacial strength test. Furthermore, in order to imitate the human physiology, mechanical tests were also performed in phosphate buffered saline (PBS) solution at 37 °C. The method herein presented provides a high control of porosity, pore size distribution and mechanical performance, thus offering the possibility to fabricate three-layered scaffolds with tailored properties by following a simple and eco-friendly route.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54595/>

Introduction

The interest in biodegradable polymers has grown in importance during the last years both in academia and in the industry, due to the rising concerns regarding plastic waste and the reduction in using non-renewable sources¹⁻⁷. In particular, biocompatible and biodegradable synthetic polymers are widespread in several biomedical application fields, such as drug controlled release^{8,9}, absorbable suture threads^{8,10}, bioprocess intensification¹¹ and tissue engineering¹².

Tissue engineering focuses on the development of devices capable to restore and maintain normal function in diseased or injured tissues. Most of the native tissues are composed by different types of cells and extracellular matrices (ECMs) in specific spatial hierarchies. For example, articular cartilage (AC) consists of different zones with varying types and orientations of collagen fibers and collagen-binding proteins. Moreover, cartilage and bone show significantly different hierarchical structures. In this context, the preparation of multilayer scaffolds with engineered properties in each layer could allow replacing heterogeneous tissues by taking into accounts all the local microenvironments of these complex systems^{13,14}.

Based on the cellular/biological and/or physical-chemical characteristics of the scaffolds, the main strategies adopted by the tissue engineering can be divided into monophasic, biphasic, and triphasic. Biphasic and triphasic approaches (BTA) use two or three different pores architectures, materials, or fillers to prepare multilayered functional devices. Furthermore, a single material can be used to achieve biphasic or triphasic devices, as long as it is possible to create a gradient in its physical properties¹².

Cell migration plays a key-role in the morphogenesis, inflammation, wound healing and tumor metastasis. Cell movement is encouraged by the presence of a gradient of chemical-physical properties from the surface to the core of the device. Therefore, biomaterials fulfilling the above discussed requirements can be helpful in studying cell migration. In addition to chemical gradients that trigger cells migration (chemotaxis), mechanical properties of cells culture substrate can also lead to cell movement (mechanotaxis)¹⁵.

The multilayer structure can also provide the tunable release of specific drugs incorporated within the polymer matrix, by changing the specific area of the layer or the amount of loaded drugs.

Over the past decade, in order to develop scaffolds possessing a discrete or continuous gradient of morphological properties, such as porosity or pore size, several approaches have been presented¹⁶⁻³¹. The most recent papers focused on the preparation of BTA by adopting: particle

leaching^{12,28,32}, gas foaming technique¹⁶, electrospinning¹⁷⁻¹⁹, layer by layer casting technique²⁰, rapid prototyping^{21,22}, thermally induced phase separation (TIPS)²², centrifugation freeze drying^{24,25}, triply periodic minimal surfaces (TPMS)²⁶, freeze casting²⁷⁻³⁰.

Within the frame of this work, we present a fast and simple route to achieve PLA-based three-layer porous scaffolds (TLS), by combining melt mixing, compression molding and salt leaching. Differently from most of the technologies commonly used for scaffold production, the strategy herein adopted can be considered fully eco-friendly, since it does not require any toxic solvent potentially dangerous for environment and for living cells and tissues³². The basic processing-structure-property relationships established in this study by analyzing both morphological features and mechanical behavior of fabricated devices provide guidance to future advances in designing multifunctional graded scaffolds with specific target properties.

Protocol

1. Scaffold Fabrication

1. Grind NaCl in a laboratory blender for 20 min and dry it on a heater at 100 °C.
2. Put the dried NaCl (45 g at time) in a sieving machine for 30 minutes at the highest available frequency without occurring in resonance. Collect six salt fractions, ranging from 500 µm to 1000 µm (M 500); from 300 µm to 500 µm (M 300); from 100 µm to 200 µm (M 100); from 90 µm to 100 (M 90); from 45 µm to 65 µm (M 45) and finally M 10 with salt particles size smaller than 45 µm as schematized in **Figure 1**.
3. Vacuum dry overnight all the materials in order to avoid hydrolytic scission during processing. For each material, select the temperature in order to maximize the degree of drying without overcoming - in the case of polymers - the glass transition. Therefore, choose T = 90 °C for PLA and T = 25 °C for PEG, T = 105 °C for NaCl.
4. Feed PLA, PEG and NaCl, with a weight percentage composition of 20/5/75 respectively, to a batch mixer operating at T = 190 °C and n = 60 rpm and then process them until achieving a constant value of torque, usually after about 10 minutes. Thereafter, rapidly collect the resulting material.
5. Prepare the mono-layers by using a laboratory press operating at 210 °C, put the blends in appropriate cylindrical molds with a diameter of 10 mm and a height of 3 mm and keep them for 60 sec at ambient pressure and 3 minutes at 180 bar. Thereafter, cool the blends at room temperature, maintaining the pressure of 180 bar.
6. Assembly of Three-layers via compression-molding
 1. Prepare each single layer in the same way as that described in (1.5) but using different molds, i.e. having a diameter of 10 mm and a height of 1 mm. Finally, obtain 6 disks having a diameter of 10 mm and a height of 1 mm, containing six different particle sizes: M 500, M 300, M 100,
 2. For assembling three-layer scaffold A (TLS A), pile up M 500, M 300 and M 100 inside the cylindrical mold and compression-mold them in a laboratory press operating at 210 °C for 60 sec at ambient pressure and 3 minutes at 180 bar and then cooled at room temperature, maintaining the pressure of 180 bar.
NOTE: Prepare the TSL B by stacking on each other M 90, M 45 and M 10 in the same molds and conduct the compression-molding operation by following the same procedure as that used for TLS A.
7. Remove the disks from the cylindrical molds and put them in a boiling demineralized water bath, without stirring. 3 hr after, remove the resulting porous structures from the bath and let them dry for 12 hr at room temperature in a chemical hood.

2. Morphological Analysis

1. Evaluate the morphologies of the scaffolds by scanning electron microscopy.
 1. Break down the samples under liquid nitrogen and then attach the samples on an aluminum stub using an adhesive carbon tape. Finally, sputter-coate with gold for 90 s under argon atmosphere before imaging in order to avoid electrostatic discharge during the test.

3. Scaffold Pore Size

1. Elaborate the images obtained by SEM analysis with an image processing software able to recognize the pore size distribution of the scaffold.
NOTE: In this work the pore size distribution analysis was carried out using a MATLAB-based software previously described³³

4. Porosity

1. Weigh the samples before leaching and evaluate the theoretical porosity according to the following expression:

$$(1) \text{Porosity}_{\text{Theoretical}}(\%) = \frac{m_{\text{NaCl}}/\rho_{\text{NaCl}} + m_{\text{PEG}}/\rho_{\text{PEG}}}{m_{\text{NaCl}}/\rho_{\text{NaCl}} + m_{\text{PEG}}/\rho_{\text{PEG}} + m_{\text{PLA}}/\rho_{\text{PLA}}} \times 100$$

NOTE: m_{NaCl} , m_{PEG} and m_{PLA} , are respectively the theoretical mass of NaCl, PEG, and PLA, by assuming homogeneous blends. The densities (ρ) of NaCl, PEG and PLA are respectively 2,16 g/cm³, 1,12 g/cm³ e 1,24 g/cm³.

2. Weigh the samples after leaching and drying in order to assess the apparent density of the samples (ρ_{scaffold}), and then evaluate the real porosity as the reciprocal of the ratio between the apparent density of the scaffold and the density of non-porous PLA by using expression (2).
NOTE: It expresses the ratio between the empty volume of the scaffold and the full volume of the scaffold (empty + full).

$$(2) \text{Porosity}(\%) = \left(1 - \frac{\rho_{\text{scaffold}}}{\rho_{\text{PLA}}}\right) \times 100$$

5. Mechanical Properties

1. Test the samples under compressive mode using a tensile machine, equipped with a 1 kN load cell. Set a constant strain rate of 1 mm min^{-1} .
2. In order to investigate the mechanical performance of the samples in physiological environment, equip the dynamometer with a bath containing (PBS), (pH = 7.4) at 37°C and perform the test with the same setup as that described at point 5.1.
 1. Before each measurement in wet environment, soak the samples with PBS in a vacuum flask for 5 min in order to let PBS fill all the pores. Thereafter, allow the scaffolds to remain in PBS at 37°C for 15 min to reach the set-point temperature.
3. Determine the interfacial adhesion strength (IAS) between the layers of TL A and TL B by using a custom-designed interfacial strength test equipment, connected to a Mechanical Testing Machine following a method described in literature^{32,34}.
 1. Fix the scaffold on the rig and ensure its correct alignment with the machine's load cell and base platen. Attach the scaffold samples to aluminum test stubs by using a high viscosity adhesive and place them into the equipment for testing.
 2. For the wet condition test, hydrate the samples in PBS for 1 hr prior to testing. Use a 1 kN load cell, under a tensile load applied at a strain rate of 1 mm min^{-1} .

NOTE: Take into account that failure may occur either at the ultimate tensile strength of one of the layers or because of delamination at the interfaces. Evaluate the IAS as the maximum strength of the stress-strain curve.

Representative Results

The influence of NaCl particle size on the pore architecture of the scaffolds was assessed qualitatively and quantitatively by investigating the morphology of the samples and calculating the pore size distribution by image analysis, respectively. **Figure 2a-f** shows SEM micrographs of mono-layered scaffolds resulting from salt-leaching of materials containing different NaCl particle sizes.

More in detail, M 500 (**Figure 2a**) showed pores with an average diameter of $500 \mu\text{m}$, probably due to the breakage of salt particles with diameter higher than $500 \mu\text{m}$ during the melt mixing. As clearly visible from the same figure, pore architecture is characterized by a low number of irregular pores, poorly interconnected, surrounded by walls of about $10 \mu\text{m}$. **Figure 2b** reports the morphology of M 300. In this case, the pores were found to exhibit an average diameter within the same range of the salt particles filled during melt mixing ($300\text{--}500 \mu\text{m}$), thus confirming that no particle breakage occurred within melt mixing process. Pore walls were found to be thinner (about $5 \mu\text{m}$) than those observed in M 500 layer. M 100, (**Figure 2c**), shows a bimodal porous structure, characterized by a heterogeneous network composed by large pores ($100\text{--}200 \mu\text{m}$) surrounded by smaller ones. This pore architecture provides a better interconnectivity and an increased pore density for volume unit, although determining a drastic thinning of walls thickness. The morphology of M 90, provided in **Figure 2d**, shows roughly cubic pores, homogeneously distributed throughout the polymer matrix, due to the smaller salt size range ($90\text{--}100 \mu\text{m}$) used in this case. Micropores, due to PEG solvation were present as micro-tunnels inside the walls that, in fact, appear very rough. SEM micrograph of M 45, shown in **Figure 2e**, displays a high density of pores, which diameters range from $45 \mu\text{m}$ to $65 \mu\text{m}$. M 10 (**Figure 2f**) displayed the highest density of pores per volume unit, an average pore size approximately equal to $20 \mu\text{m}$, with a high degree of interconnection and very thin ($<1 \mu\text{m}$) walls.

Figure 3a, a', a'' displays a cross-section of TLS A, after the leaching process, at different magnifications. In **Figure 3a** it is possible to clearly identify the three layers, each one characterized by different average pore size, while the panels **a'** and **a''** refer to M 100-M 300 and M 300-M 500 interface regions, respectively. As clearly visible, the whole device do not present any internal cleavages nor matrix discontinuity between the different layers. Analogously, TLS B and related interfaces are reported in **Figure 3b, b', b''**. The images revealed a morphology similar to that of TLS A. Indeed, three layers with pores of different sizes can be easily recognized (panel b), whereas both M 10-M 45 (panel b') and M 45-M 90 (panel b'') interfacial regions exhibited no flaking nor discontinuity. As expected, each single layer retains the same pore architecture after assembly and leaching steps.

Table 1 reports the compressive elastic moduli of the materials, measured in air (dry) and in PBS (wet) environment. This property was found to follow a monotonic increase with the mean pore size. The elastic moduli of the final devices are mainly determined by their respective weaker layer (*i.e.* M 100 for TLS A and M 10 for TLS B) for both the TLSs investigated. **Table 2** reports IAS for TLS A and TLS B in dry and wet environment. No interlayer delamination phenomena were observed, since the failure always occurred in the middle of the weakest layers of TLS A (M 100) and TLS B (M 10). TLS A displayed the best IAS performance.

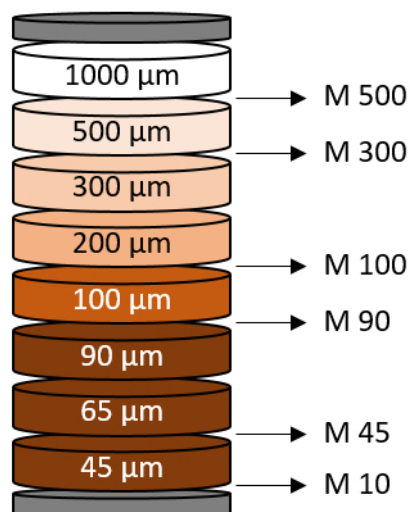


Figure 1: Schematics of NaCl granulometry. Granulometry of sieved NaCl particles and corresponding sample codes.

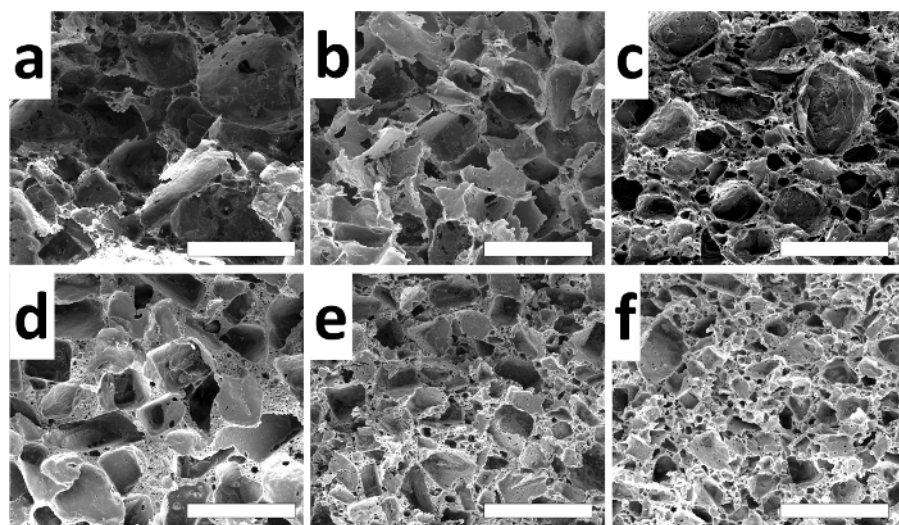


Figure 2: Morphology of monolayer scaffolds. SEM micrographs of monolayer scaffolds characterized by different pore size distributions: M 500 (a), Scale bar = 400 μm ; M 300 (b), Scale bar = 400 μm ; M 100 (c), Scale bar = 400 μm ; M 90 (d), Scale bar = 200 μm ; M 45 (e), Scale bar = 400 μm and M 10 (f), Scale bar = 100 μm . [Please click here to view a larger version of this figure.](#)

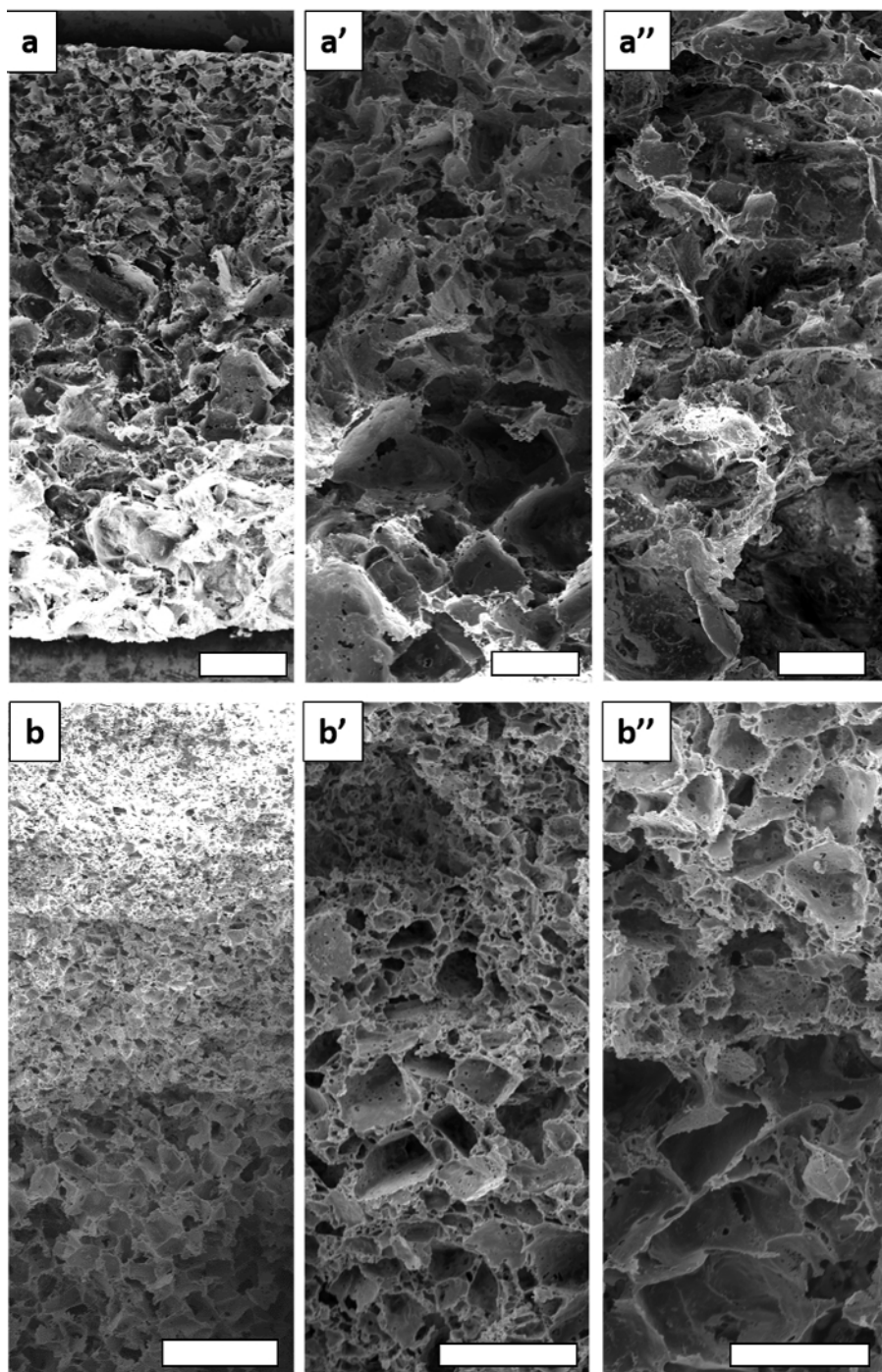


Figure 3: Morphology of three-layer scaffolds. (a, a', a'') SEM micrographs of three-layered scaffold type A (TLS A): (a) entire cross section, Scale bar = 500 μ m; (a') M 100-M 300 interface, Scale bar = 250 μ m; (a'') M 300-M 500 interface, Scale bar = 250 μ m. (b, b', b'') SEM micrographs of three-layered scaffold type B (TLS B): (b) entire cross section, scale bar 500 μ m; (b') M 10-M 45 interface, Scale bar = 100 μ m; (b'') M 45-M 90 interface, Scale bar = 100 μ m. [Please click here to view a larger version of this figure.](#)

Sample code	Dry - E (MPa)	Wet - E (MPa)
M 500	40.33 ± 6.04	33.23 ± 4.96
M 300	37.62 ± 6.89	31.42 ± 5.83
M 100	32.12 ± 5.11	28.03 ± 4.04
M 90	30.87 ± 4.93	26.91 ± 3.79
M 45	25.36 ± 5.82	22.83 ± 5.01
M 10	21.76 ± 3.91	19.87 ± 3.93
TL A	33.08 ± 5.21	29.55 ± 4.09
TL B	22.31 ± 5.46	20.54 ± 3.87

Table 1: Compressive mechanical results. Compressive Young modulus of mono and three-layered scaffolds with different pore sizes in wet and dry environment. Values are given as means ± SD.

Sample code	Dry - IAS (kPa)	Wet - IAS (kPa)
TL A	350.8 ± 51.2	299.6 ± 35.1
TL B	262.3 ± 62.2	220.5 ± 31.3

Table 2: Scaffold interfacial adhesion strength. Interfacial adhesion strength test results for TL A and TL B in dry and wet condition. Values are given as means ± SD.

Discussion

The first critical step is the optimization of sieving efficiency. The high control of the NaCl particle size is fundamental for preparing scaffold with desired pore size distribution. Another critical step is avoiding the fracture of the thin PLA monolayers during the sample extraction from the mold. The image processing analysis might not be representative of the whole device.

During tensile tests, the sample can tear away from the equipment.

Prior to sieving step, ascertain that the salt have been well dried in order to get a higher control of the NaCl particle size. This issue is important especially for the smallest salt particle size, due to its extremely high hygroscopicity. Prior to compression-molding step, apply a thin Teflon spray-coating onto the molds in order to facilitate the sample removal from the molds. The image processing analysis must be carried out by considering different images taken from different regions of the scaffold to be sure that they are representative of the whole device. Finally, prior to tensile tests (especially those carried out in wet environment), carefully verify the adhesive to sample adhesion.

The principal limitation of the technique lies in the impossibility to obtain a continuous gradient of the pore size. Indeed, the method herein described allows achieving a discrete gradient of pore size, since it is based on the assembling of different layers. In most cases, a well-defined multilayer scaffold can be preferred to a continuously graded one but not ever. This limitation may be partially exceeded by increasing the number of layers, which in turns would result in thinner layers, obviously more difficult to handle.

Differently from many other production technologies, the strategy here adopted can be considered eco-friendly, since it does not require any toxic solvent potentially dangerous for environment and for living cells and tissues. Furthermore, particulate leaching provides a high control of both pore size and porosity by tuning respectively the size and the amount of the NaCl blended with the PLA.

Future advances of this technique rely on the possibility to gather layers presenting other chemo-physical differences. For instance, one can assemble different biopolymers or reinforce each layer with different nanoparticles, such as hydroxyapatite²⁸, nanocellulose²⁷, graphene³⁵ or its derivatives^{9,36,37} in order to provide even further functionalities³⁸. In fact, this method ensures a high control thus allowing to easily tune each region of multilayer scaffold. This challenge play a crucial role in the ITE, since the presence of multi-phasic and/or anisotropic bio-structures that gradually change from one tissue to another are typical features of interface tissues, such as ligament-to-bone, tendon-to-bone and cartilage-to-bone.

Disclosures

The authors declare that they have no competing financial interests.

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