CHEMICAL ENGINEERING TRANSACTIONS
VOL. 64, 2018
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Antonio Marzocchella, Tajalli Keshavarz

ISBN 978-88-95608-56-3; **ISSN** 2283-9216
DOI: 10.3303/CET1864033

Evaluation of Hydroxyapatite distribution in a Poly-L-Lactic Acid (PLLA) scaffolds via Micro Computed Tomography (*μCT*)

Ilenia Vitrano*[a], Francesco Carfì Pavia[a,b], Gioacchino Conoscenti[a], Maria Elena Lombardo[a], Vincenzo La Carrubba[a,b], Valerio Brucato[a,b]

[a]DICAM, University of Palermo, Viale delle Scienze building 8, 90128 Palermo
[b]ATeN center, CHAB, University of Palermo, Viale delle Scienze building 18, 90128 Palermo
ilenia.vitrano@unipa.it

Bone Tissue Engineering offers promising clinical alternative substitutes for bone defects, focusing on the use of polymer/ceramic composites. Hydroxyapatite (HA), a bioactive ceramic, has been implemented in bone substitution and regeneration due to its biocompatibility, osteoconductivity and close resemblance to the mineralized phase of human bone. Several techniques have been adopted to characterize composite scaffolds in terms of morphology, pore size and interconnection, filler content and distribution, but most of them are destructives. In this work, composite Poly-L-Lactic Acid (PLLA)-HA scaffolds (17.6 mm diameter and 35.7 mm height) were prepared via Thermally Induced Phase Separation (TIPS) by using a ternary PLLA-dioxane-water solution (polymer-solvent-nonsolvent). Two different concentrations (10% and 20% wt/wt filler/polymer ratio) of HA particles were chosen. The samples were characterized via Scanning Electron Microscopy (SEM) and Microcomputed Tomography (μCT), a non-invasive technique capable to analyze the internal structure of the sample. After the μCT reconstruction, an investigation of the distribution and average sizes of the filler was carried out through a 3D analysis software (CTAn). The obtained results demonstrated the possibility to analyze the internal distribution of the particles via a non-invasive technique; furthermore a homogeneous HA content and a uniform size distribution in the whole sample was observed, which allows one to assess that the scaffold production technique does not create neither a filler gradient nor a particle sedimentation.

1. Introduction

Bone is a complex porous composite, living, constantly changing tissue. It has unique properties of remodelling to adapt its microstructure to external mechanical stress (Mathieu et al., 2006). Nowdays Tissue Engineering offers valid alternatives to regenerate damaged and diseased tissues or organs in order to overcome limitations of traditional autografts and allografts, such as risks of immune rejection, pathogen transfer and inflammation. Growing proofs highlight that Tissue Engineering approaches have remarkable potential for control of biomaterial properties to meet the biomechanical and biological requirements of complex tissues (Boerckel et al., 2014). Particularly, bone tissue engineering provides an interesting new approach for bone repair, due to its ability to involve different combinations of biomaterials and biologics such as proteins, cells, and growth factors inducing bone regeneration. Numerous types of biodegradable scaffolds have been fabricated and investigated during the years in the search for effective bone graft substitutes. Synthetic materials, such as poly(lactideco-glycolide) (PLGA), polyglycolide (PGA) and polylactide (PLA) (Conoscenti et al., 2017) have been used either alone or in combination with naturally derived materials including collagen, chitosan, starch and silk fibroin (Lee et al., 2006). Polymer-based scaffolds must often be surface modified or combined with bioactive fillers, for example ceramics bioactive glass or hydroxyapatite (HA), to achieve the desired mix of properties (Healy and Guldberg, 2007, Ghersi et al., 2016). The bioactive ceramic HA, since the ‘80s, has been implemented in bone substitution and regeneration due to its property of biocompatibility, osteoconductivity and similarity to the mineralized phase of human bone, stimulating the

Please cite this article as: Vitrano I., Carfi Pavia F., Conoscenti G., Lombardo M.E., La Carrubba V., Brucato V., 2018, Evaluation of hydroxyapatite distribution in a poly-l-lactic acid (plla) scaffolds via micro computed tomography (μct), Chemical Engineering Transactions, 64, 193-198 DOI: 10.3303/CET1864033

formation of new tissue. Scaffold composition and morphology play a key role in cell attachment, proliferation, and differentiation (Ho and Hutmacher, 2006). Kim et al. (2006) used the gas forming-particulate leaching method to fabricate PLGA/HA composite scaffolds for bone tissue engineering. To increase the HA exposure to the scaffold surface, they used HA particles approximately 100 nm in size rather than micro-sized particles. Over the years, different techniques have been adopted to characterize the scaffolds in terms of morphology, pore size and interconnection, filler content, distribution and size, but most of them are destructives. Among these techniques Thermal Gravimetric Analysis (TGA) has been often adopted to quantify the amount of filler present in a matrix. There are many studies about the characterization of ceramics scaffolds or polymer matrices by using destructive techniques e.g. Renghini et al. (2008) and E. Dìaz et al. (2014). Furthermore, to establish how the hydroxyapatite modifies the structural properties of the scaffolds, mechanical testing are also to be carried out, such as compression tests to evaluate foam mechanical anisotropy in compression (Mathieu et al., 2006). Non-destructive evaluation of microstructural characteristics is necessary for scaffold optimization, especially in terms of particle size and distribution. X-ray microtomography allows one to obtain scaffolds images in three dimensions and to investigate the inner structure, sheding light on any defects and imperfections that would cause problems in the regeneration of bone tissue, without damaging the construct (Boerckel et al., 2014). This work proposes the use of micro-computed tomography (μCT) as a non-invasive analysis technique for morphological and morphometric characterization of PLLA porous scaffolds containing HA, which can be used as matrices for bone regeneration. By exploiting the advantages of this methodology, we investigated the three-dimensional internal structure of composite scaffolds (PLLA-HA) produced via Thermally Induced Phase Separation (TIPS), verifying how similar the structure attained is to natural bone. Moreover, a quantitative analysis on HA particles allowed us to obtain thorough data about the volumetric distribution of nano-particles within the polymer matrix.

2. Materials and Methods

2.1 Scaffolds Preparation via Thermally Induced Phase Separation (TIPS)

PLLA/HA composite scaffolds were prepared by via TIPS using a ternary solution polymer/solvent/nonsolvent adding different concentration of hydroxyapatite particles. This method is based on the liquid-liquid demixing in a polymer-lean and a polymer-rich phase, which occurs when the system is maintained under cloud point temperature, depending on polymer percent and the water/dioxane ratio (Mannella et al., 2014). In particular, we chose a ternary system PLLA-dioxane-water, with 87/13 wt/wt as dioxane/water ratio and PLLA at 4% wt. First the polymer was dissolved into 1,4-dioxane by stirring at a temperature of 120°C. After its complete dissolution the right amount of distilled water was added. In a second step, we added to the prepared solution two different percentages of HA (10% and 20% wt/wt). In order to reduce the formation of aggregates, blending by ultrasonic stirring was carried out for 30 min. Cylindrical scaffolds used in this study were obtained by pouring 5 mL of the solution in a High Density Poly-Ethylene (HDPE) cylindrical sample holder (inner diameter 17.6 mm and 35.7 mm height). Then, the sample holder was immersed into a thermostatic bath at 30°C for 15 minutes in order to obtain a porous structure. Finally, the system was frozen at - 20°C in order to stop the process.

2.2 Scaffold Characterization

The structure and morphology of composite scaffolds were examined by SEM and μCT. SEM analysis was limited to the observation of cross sections of the sample (2D image), whereas the μCT was employed to carry out qualitative and quantitative analysis and to visualize the three-dimensional internal structure of composite scaffolds. Specifically, the software for 3D analysis allowed us to valuate HA nano-particles size and their three-dimensional distribution into the polymer matrix. Quantitative analyses were performed by following two different methods, nominally method A and method B. In the first one, we considered a Volume of Interest (VOI) consisting of 600 layers (Figure 1A). On the other hand, in the method B, for each cylindrical scaffolds, three representative regions of 300 layers were obtained from Top, Middle and Bottom regions (Figure 1B).

2.2.1 Scanning Electron Microscopy (SEM)

The bulk morphology of the scaffolds was examined by using scanning electron microscope (SEM) Phenom Pro-X (Phenom-World, Netherlands). Samples were placed onto an aluminum stub covered by conductive carbon tabs and observed at 10 kV.

2.2.2 Microcomputed Tomography (μCT)

μCT scanner employed was a Skyscan 1272 (Bruker), kindly made available by ATen Center, University of Palermo, with penetrative X-rays of 40 kV and an intensity of 250 mA without filters. As a result of this energy

level we had an appropriate contrast for the reconstruction of polymer scaffolds highlighting the dimension and distribution of nHA particles. The Skyscan 1272 allows small objects to be scanned with a spatial resolution of the micron order, which corresponds roughly to a $1x10^{-9}$ mm^3 size voxel. The system reconstructs the sample in its three-dimensionality and also offers the possibility of having morphometric information on the observed sample, an useful information for the characterization of biomaterials. The instrument is essentially composed of a microfocal X-ray source; a Charge Coupled Device (CCD) X-ray detector that captures the transmitted radiation emitted from the object; a rotating swing holder to allow rotation of the object during tomographic series acquisition and a camera to verify the correct positioning of the specimen. The instrument is controlled by a dual-processor PC with software for three-dimensional reconstruction of the images and for its calculation of the morphometric parameters. Each cylindrical sample was laid on a rotating support and the images were acquired rotating the specimen to 180° with a rotation step of 0.1°. The X-ray scans were acquired in high-resolution mode getting a value of pixel size of 3.75 µm. The scanning dataset obtained after the acquisition step consisted of images in 16-bit tiff format (3,238×4,904 pixels). The whole process (acquisition, reconstruction and analysis) was performed by using Bruker's software. After sample scanning the acquired images were reconstructed by software N-Recon, used for elimination of artifacts and reduction of noise in each slice. The program returns the reconstructed images of all the horizontal sample sections called "Raw images" in 8-bit depth with 256 gray levels (Figure 4). Thus, image datasets were visualized with software Vox-CT to obtain a three-dimensional view of the scaffold. Through a visualization in the 3D space it is possible to identify surface sections having a direct comparison with SEM surface images. After morphological, a morphometric characterization was performed on scaffolds with hydroxyapatite. Three dimensional quantitative analysis were performed using software CTAn. These quantitative analyses were performed on binary images obtained by applying on the raw image an appropriate thresholding value. The threshold range was manually set for each single sample, in order to highlight only the HA particles (Figure 5). Finally, 3D quantitative analysis gave information about the total volume of HA particles and their average size.

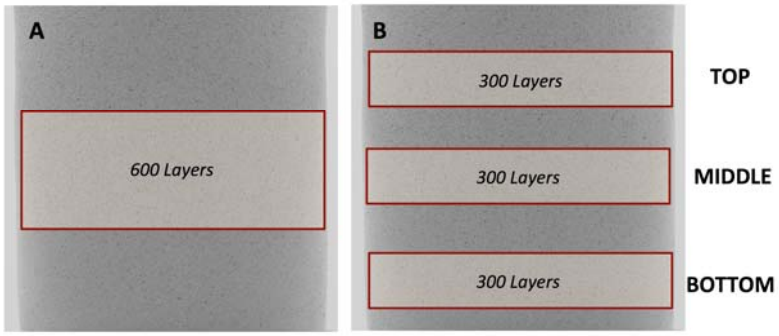

Figure 1: VOIs for quantitative analysis: Method (A) 600 layers on the middle region of the sample; Method (B) Three regions of 300 layers

3. Results and discussion

3.1 Qualitative analysis

SEM micrographs of samples (Figure 2) show a structure characterized by interconnected pores and a good polymer/filler interpenetration, i.e. the walls of the pores were constituted by the polymer matrix with nanoparticles distributed homogeneously. In order to appreciate in detail sample internal morphology qualitative and quantitative analysis on PLLA/HA composite scaffolds, Micro X-ray Computed Tomography was employed. To this end, a three-dimensional reconstruction of scaffolds was obtained, following a rigorous selection of acquisition parameters by a visualization software CTVox. In 3D reconstruction shown below (Figure 3A and 3B) we can distinguish the HA particles from the polymer matrix for their higher density. Similarly to SEM images, the three-dimensional viewing reveals a homogeneous HA distribution, also in the presence of different filler concentrations.

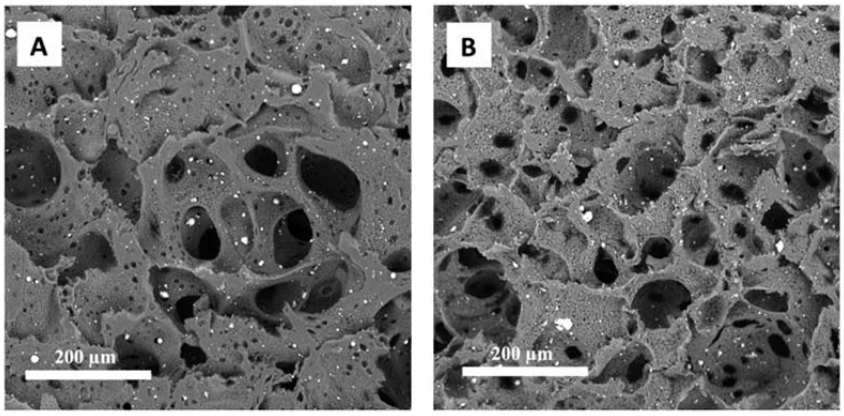

Figure 2: SEM images of scaffolds HA/PLLA prepared via TIPS 30 °C 15 min with a concentration of HA (A) 10% wt and (B) 20% wt

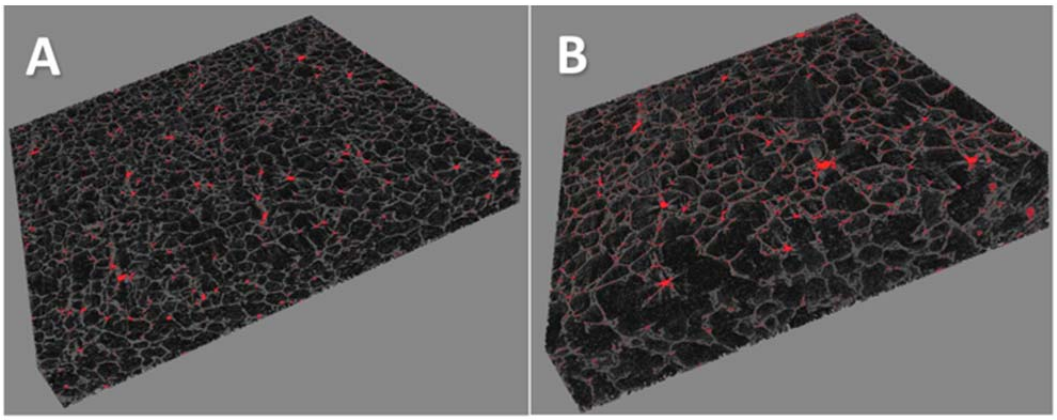

Figure 3: 3D Reconstruction of composite scaffold: (A) PLLA/HA 10 %wt; (B) PLLA/HA 20 %wt

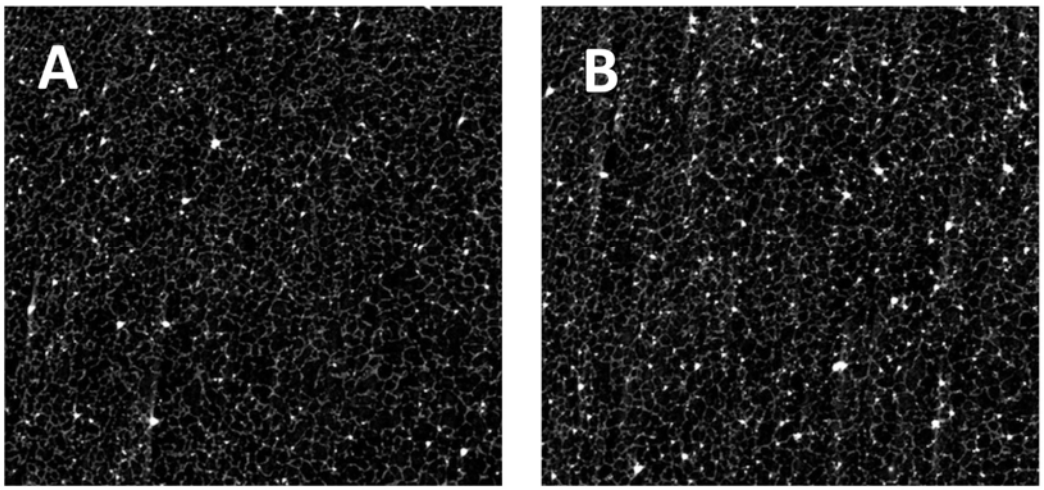

Figure 4: Raw images: A) PLLA/HA 10 %wt ; B) PLLA/HA 20 %wt

3.2 Quantitative analysis

3D quantitative analysis onto the set of binarized images shown in Figure 5 allowed to estimate the volume percentage of hydroxyapatite with respect to VOI and the average size of HA particles with their relative distribution. As described above, two different methods were tested to carry out the analysis. In Table 1 we

report the results about the percent HA particles volume defined as the ratio between object volume and Total VOI. The value show in below table are obtained through the two methods.

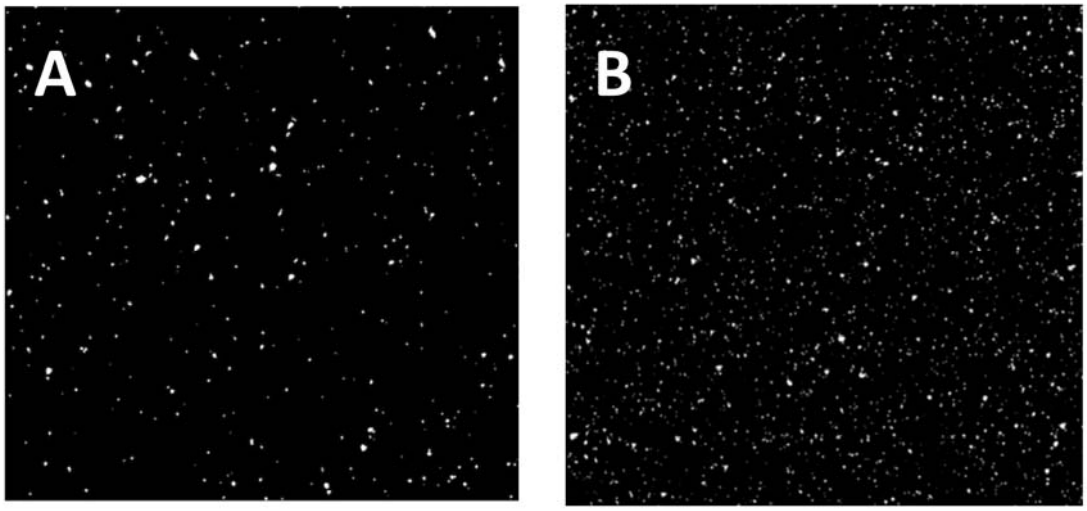

Figure 5: Binarized images: A) PLLA/HA 10 %wt; B) PLLA/HA 20 %wt

The data shows as the analysis carried out following both method A and method B produce very similar values in terms of percent object volume. Especially the value obtained in both samples with method A are around similar to the average value in the three regions by method B. The main advantage obtained by using Method B was to speed up considerably the computational time. Furthermore, thanks to this latter method we can observe an almost uniform content of filler in the three sections of the scaffold. So the possibility to perform quantitative analyses according to method B plays a key role in confirming a hypothesis barely detectable through any other characterization techniques: the sedimentation of HA particles does not take place significantly during the scaffold preparation process. Furthermore, from the analysis, mean particle diameter distribution was obtained. The histogram in Figure 6 show the percent object volume of the size of the particles in the scaffold prepared with 20 %wt HA. The data has revealed that for all the samples examined The HA particles have a mean size in the range of 11 to 18 μm. The same result was obtained for the samples prepared with 10 %wt HA.

Table 1: Percent Object Volume in the considered VOI

Method A (600 layers)			Method B (300 layers)	
10% HA	20% HA		10% HA	20% HA
		TOP	0.70	1.86
0.65	1.67	MIDDLE	0.67	1.71
		BOTTOM	0.68	1.61

This work proves the main benefits provided by use of μCT as an innovative scaffold characterization. The possibility to exploit the micro-CT imaging may be very useful to carry out qualitative and quantitative analysis in a non-destructive way; this could allow an effective pre-screening method for the validation of the morphological features of the sample. SEM analysis allowed to investigate the morphology and distribution of HA only in 2D images. Thanks to μCT we are able to enquire into 3D structure with acquisition and 3D Reconstruction step. In addition, the quantitative analysis carried out separately on the three-VOI allowed one to calculate HA volume percentage in each sample and average size of particles. The results obtained showed a very similar HA content in the different VOIs, which implies the absence of particles sedimentation and consequently a homogeneous HA distribution in the whole scaffold. Moreover, it was observed that all VOIs present the same size distribution of HA particles. This result allows one to infer that during the TIPS process, HA particles are efficiently distributed within the matrix without generating filler gradients.

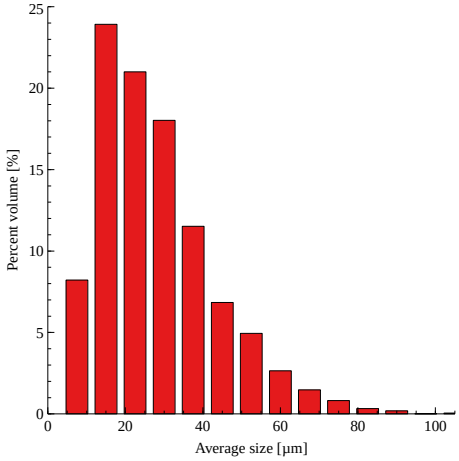

Figure 6: Volume Percent of particles vs. average size (PLLA/HA scaffold 20 %wt)

4. Conclusion

In the present study, a porous scaffold PLLA/HA was successfully fabricated via TIPS with different hydroxyapatite concentration. For characterization of the structure a computed tomography was employed. 3D reconstruction via µCT lighted up on the interaction of the filler with the polymer solution during the TIPS. Also quantitative analysis of HA particles allowed to estimate the average particle size in the range of 11-18 µm. Moreover, the quantification of the HA particles volume along the axial axis of the scaffolds showed that no filler concentration gradient was found as a homogeneous distribution if the particles was observed. To sum up, it can be concluded that the sedimentation process does not take place during the production process.

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