



UNIVERSITÀ DEGLI STUDI DI PALERMO

Dottorato di Ricerca in Ingegneria Civile, Ambientale e dei Materiali
Dipartimento di Ingegneria
ING-IND/24

ADVANCED TECHNIQUES FOR THE IMPROVEMENT OF POLYMERIC COMPOSITE SCAFFOLDS FOR BONE TISSUE ENGINEERING

USO DI TECNICHE AVANZATE PER LA OTTIMIZZAZIONE STRUTTURALE E
FUNZIONALE DI SCAFFOLD COMPOSITI POLIMERO-CARICA INORGANICA PER
LA RIGENERAZIONE OSSEA

IL DOTTORE
ING. ILENIA VITRANO

IL COORDINATORE
PROF. ANTONINA PIRROTTA

IL TUTOR
PROF. VALERIO M.B. BRUCATO

CO TUTOR
PROF. VINCENZO LA CARRUBBA

IL TUTOR ESTERO
PROF. ALDO BOCCACCINI

DOTT. FRANCESCO CARFI' PAVIA

IL TUTOR AZIENDALE
PROF. GIULIO GHERSI



UNIVERSITÀ DEGLI STUDI DI PALERMO

Dottorato di Ricerca in Ingegneria Civile, Ambientale e dei Materiali

Dipartimento di Ingegneria

ING-IND/24

ADVANCED TECHNIQUES FOR THE IMPROVEMENT OF POLYMERIC COMPOSITE SCAFFOLDS FOR BONE TISSUE ENGINEERING

**USO DI TECNICHE AVANZATE PER LA OTTIMIZZAZIONE STRUTTURALE E
FUNZIONALE DI SCAFFOLD COMPOSITI POLIMERO-CARICA INORGANICA PER
LA RIGENERAZIONE OSSEA**

**IL DOTTORE
ING. ILENIA VITRANO**

Ilenia Vitrano

**IL TUTOR
PROF. VALERIO M.B. BRUCATO**

Valerio M.B. Brucato

**IL TUTOR ESTERO
PROF. ALDO BOCCACCINI**

**IL TUTOR AZIENDALE
PROF. GIULIO GHERSI**

**IL COORDINATORE
PROF. ANTONINA PIRROTTA**

**CO TUTOR
PROF. VINCENZO LA CARRUBBA**

DOTT. FRANCESCO CARFI' PAVIA

Abstract

Bone tissue engineering is a promising field of research, which aims to develop biological substitutes for repairing and/or replacing damaged tissues. Recently, the need to rebuild damaged bone tissue following severe trauma, congenital malformations or removal of bone tumors is increasingly growing. Tissue Engineering (TE) offers valid alternatives to traditional surgical strategies in order to overcome limitations of traditional autografts and allografts, such as risk of immune rejection, pathogen transfer and inflammation. The regeneration of tissues is mediated by a specific biocompatible scaffold.

The aim of this Ph.D. project is to investigate the possibility to mimic as closely as possible the bone tissue and ligaments in terms of morphology and properties, by introducing composite scaffold (polymer + ceramic filler) in order to improve the mechanical properties compared to the neat polymer, and better structural integrity and flexibility than brittle ceramics.

In fact, the combination of ceramic and polymer could provide reinforced porous structures with enhanced bioactivity and controlled reabsorption rates. The fabrication technique and the biomaterial that are used to produce the scaffold play a crucial role in the ability to support cell growth and diffusion of both cells and nutrients. Given that the scaffold should strictly mimic the structure of the tissue to regenerate, it should have mechanical properties matching those of the tissue at the implantation site, an appropriate

porosity (in terms of pore size and pore distribution) and the capability of being sterilizable without causing any chemical change.

Two efficient and versatile techniques have been adopted for the realization of composite scaffolds: thermally induced phase separation (TIPS) and diffusion induced phase separation (DIPS). The first is a technique that allows to obtain porous structures with a high degree of interconnection, while the second allows the production of porous scaffolds with a homogeneous microporous structure of different shapes (tubular or film). Among the biocompatible polymeric materials extensively studied for biomedical applications, there are the poly (α -hydroxy acids), approved by the Food and Drug Administration (FDA) of the United States. In particular the poly- (L-lactic acid) (PLLA) has attracted most interest thanks to its suitable mechanical properties, low toxicity and predictable biodegradation kinetics. Furthermore, PLLA can be easily processed and its mechanical and degradation properties can be modulated according to the target application. Other studies have shown that the use of materials such as hydroxyapatite and the bioactive glass, have positive effects since they induce cell differentiation and also present osteoinductive activity. In bone regeneration, angiogenesis plays a central role by providing the functional connection between the grafting material and surrounding host tissues. So, the interest in the use of bioactive glass has strongly increased thanks to its angiogenic potential. However, these ceramics highlight some limitations that do not make them suitable for being used alone, especially for applications aimed at replacing more mechanically stressed portions of bone. For this reason, these materials are increasingly used as fillers in polymeric matrices to achieve

the desired mix of properties. Scaffolds in poly-L-lactic acid (PLLA) containing hydroxyapatite (HA) and/or bioactive glass (BG) nanoparticles have been produced in different shapes and with different concentrations of ceramics based on the final application (bone or ligaments).

For bone regeneration were proposed PLLA-HA, PLLA-HA-BG and PLLA-BG scaffold produced via TIPS. The contribute given by this work was used an easy and versatile technique to get support with 95% of porosity than around 90% that can be obtained in other fabrication process. In the years different methods 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. Furthermore, this work aims at making use of a Micro-Computed Tomography (μ CT) as non-invasive analysis technique for morphological and morphometric characterization. The results confirm that it is possible estimate porosity, pores size and particle distribution in a single step: an image analysis of μ CT scan.

The osteoblast cell cultured on PLLA/HA has shown a good proliferation until 49 days of culture where the scaffold totally was colonized, instead according to other studies the osteoblast differentiation started after 21 days.

Here to overcome a problem joining to the BG integration in polymer mesh produced via TIPS, was proposed an alternative to the standard technique. A possibility to pretreated the BG was evaluate before the suspension into solution. This confirms that the pre-treatment contributes to ions release increase integration (Young module increase

until 5 MPa) without alter BG bioactivity. Furthermore, the borate BG was found stimulating the VEGF expression and the angiogenic process.

At the end, a new approach in anterior cruciate ligaments regeneration has been proposed. Using the tubular scaffolds different from that produced with different technique helps the anterior cruciate ligaments growth and the cell alignment.

The thesis is composed of eight chapter, summarized below:

Chapter 1: an introduction and overview on the bone and ligaments injures. Comparing between traditional and tissue engineering approach.

Chapter 2: The aim of this work was highlighted and the material and technique proposed as new strategies

Chapter 3: Characterization technique used to obtain a complete set of data about porosity, pores size, particles distribution.

Chapter 4: the behaviour of osteoblasts in the produced PLLA/HA matrices was evaluated by means of cell viability and differentiation tests. This study allowed us to determine how the concentration of HA affects the differentiation process.

Chapter 5: PLLA/BGs scaffold were produced. In vitro bioactivity test of PLLA/BGs membranes in a simulated body fluid solution (SBF) is illustrated.

Chapter 6: the possibility to produce composite PLLA-HA-BG scaffolds via TIPS was investigated. Morphological investigations and in-vitro bioactivity tests were performed.

Chapter 7: development of a suitable technique for *in vitro* seeding of lapine ACL fibroblasts into tubular-shaped instructive PLLA scaffolds, supplemented or not with bioglass, produced via DIPS.

Sommario

L'ingegneria del tessuto osseo è un campo di ricerca promettente, che mira a sviluppare sostituti biologici per la riparazione dei tessuti danneggiati. Negli ultimi anni, è sempre più crescente la necessità di ricostruire un tessuto osseo danneggiato a seguito di gravi traumi, malformazioni congenite o asportazione di tumori ossei.

L'ingegneria tissutale offre valide alternative alle strategie chirurgiche tradizionali al fine di superare i limiti degli autotrapianti e degli allotrapianti tradizionali, come il rischio di rigetto immunitario, trasferimento di agenti patogeni e infiammazione.

La rigenerazione dei tessuti, come illustrato nel *capitolo 1*, è mediata da specifici scaffold biocompatibili, definiti come dispositivi 3D sintetici biodegradabili che emulano la matrice extracellulare (ECM), la cui funzione principale è quella di fornire un supporto temporaneo alle strutture dei tessuti. Gli scaffold ideali dovrebbero promuovere le interazioni cellulari e lo sviluppo dei tessuti e possedere proprietà meccaniche e fisiche adeguate.

L'obiettivo del progetto di ricerca ha previsto il miglioramento delle conoscenze sulla produzione ed uso di scaffold compositi polimero-carica inorganica da utilizzare come sostituti ossei biodegradabili capaci di sostenere e guidare la rigenerazione del tessuto. In effetti, la combinazione di ceramica e polimero migliora sia le proprietà meccaniche del polimero puro, sia l'integrità strutturale e flessibilità dei ceramici, fornendo strutture porose rinforzate con una maggiore bioattività e tassi di riassorbimento controllati. La tecnica di fabbricazione e il biomateriale utilizzati per produrre gli scaffold

svolgono un ruolo cruciale nella capacità di supportare la crescita cellulare e la diffusione di cellule e sostanze nutritive. Dato che l'impalcatura dovrebbe imitare rigorosamente la struttura del tessuto da rigenerare, dovrebbe avere proprietà meccaniche corrispondenti a quelle del tessuto nel sito di impianto, un'adeguata porosità (in termini di dimensione dei pori e distribuzione dei pori) e la capacità di essere sterilizzabile senza causare nessun cambiamento chimico.

Due tecniche efficienti e versatili, presentate nel secondo capitolo, sono state adottate per la realizzazione di scaffold compositi: la separazione di fase indotta termicamente (TIPS) e la separazione di fase indotta per diffusione (DIPS). Come riportato in letteratura, la prima è una tecnica che consente di ottenere strutture porose con un alto grado di interconnessione, la seconda invece consente di produrre scaffold porosi con struttura omogenea microporosa di differenti forme (tubulari o film). Tra i materiali polimerici biocompatibili maggiormente studiati per applicazioni biomediche, ci sono i poli (α -idrossiacidi), approvati dalla Food and Drug Administration (FDA) degli Stati Uniti. In particolare il poli-(acido L-lattico) (PLLA) ha suscitato maggiore interesse grazie alle sue proprietà meccaniche adeguate, bassa tossicità e cinetica di biodegradazione prevedibile. Inoltre, il PLLA può essere facilmente elaborato e le sue proprietà meccaniche e di degradazione possono essere modulate in funzione dell'applicazione di destinazione. Tuttavia, per realizzare la rigenerazione ossea l'uso di un polimero da solo non fornisce la resistenza adeguata. Altri studi hanno mostrato che l'impiego di materiali ceramici come l'idrossiapatite e il biovetro, abbiano degli effetti positivi dal momento che inducono il differenziamento cellulare e presentano anche

attività osteoinduttiva. Nella rigenerazione del tessuto osseo, l'angiogenesi svolge un ruolo centrale fornendo la connessione funzionale tra il materiale innestato e i tessuti ospiti circostanti. Per questo motivo, grazie al suo potenziale angiogenico l'interesse per biovetro è in continuo aumento. Tuttavia, questi materiali ceramici evidenziano alcune limitazioni che non li rendono adatti per applicazioni volte a sostituire porzioni di ossa maggiormente sollecitate meccanicamente. Per questo motivo, essi sono sempre più utilizzati come riempitivi in matrici polimeriche per ottenere il mix desiderato di proprietà.

Scaffold in acido poli-L-lattico (PLLA) contenenti nanoparticelle di idrossiapatite (HA) e/o vetro bioattivo (BG) sono stati prodotti di diversa forma e con diverse concentrazioni di ceramici in base all'applicazione finale (osso o legamenti). La caratterizzazione degli scaffold, nonché la determinazione della composizione e della morfologia, svolgono un ruolo chiave nell'attaccamento, proliferazione e differenziazione delle cellule. Nel corso degli anni sono state adottate diverse tecniche, riportate nel *terzo capitolo*, per investigare la morfologia, dimensione dei pori e interconnessione, contenuto di riempitivo, distribuzione e dimensione, ma la maggior parte di esse sono distruttive. Inoltre, questo lavoro mira a utilizzare una micro-tomografia (μ CT) come tecnica di analisi non invasiva per la caratterizzazione morfologica e morfo-metrica. Le principali caratteristiche degli scaffold compositi, il comportamento meccanico e la bioattività in ambienti a pH fisiologico sono stati studiati nei successivi capitoli. In particolare, nel *capitolo 4* il comportamento degli osteoblasti in matrici PLLA/HA prodotte è stato

valutato mediante test di vitalità e differenziamento cellulare. Questo studio ha permesso di determinare come la concentrazione di HA influisce sul processo di differenziamento. Nel *capitolo 5*, PLLA/BG scaffold furono prodotti via TIPS e DIPS e successivamente caratterizzati. Nel primo studio, un metodo per migliorare l'integrazione di BG nella matrice polimerica è stato investigato e successivamente valutato il comportamento meccanico delle matrici ottenute. Nel secondo, il test di bioattività di membrane PLLA/BG in una soluzione simulata di fluidi corporei (SBF) è stato condotto. Inoltre, la biocompatibilità è stata studiata con cellule stromali del midollo osseo (ST2) mediante test indiretti, al fine di valutare i possibili effetti citotossici dei prodotti di dissoluzione dei materiali. Studi recenti suggeriscono che scaffold compositi basati su polimeri biodegradabili contenenti cariche minerali sono impianti che mostrano un grande potenziale osteoinduttivo. Nel *capitolo 6* è stata valutata la possibilità di produrre scaffold compositi PLLA-HA-BG via TIPS. Studi morfologici e di bioattività sono stati condotti.

Infine, un nuovo approccio nella rigenerazione dei legamenti crociati anteriori è stato proposto nel *capitolo 7*. L'obiettivo è stato quello di sviluppare una tecnica di seeding di fibroblasti di legamenti crociati anteriori di coniglio all'interno di scaffold tubulari in PLLA con o senza l'aggiunta di BG e prodotti via DIPS. La vitalità cellulare e la morfologia furono determinate mediante colorazione live-dead e Ematossilina/Eosina.

Acknowledgements

Il PhD è stato un percorso lungo tre anni, ricco di incontri, esperienze, viaggi, collaborazioni e altamente formativo per la mia crescita personale e professionale.

Per prima cosa volevo ringraziare i miei tutor Prof. V. Brucato and V. La Carrubba che sono stati fondamentali per la mia crescita scientifica e professionale. Un meritato grazie va al Dott. Francesco Carfi Pavia, il quale è stato un fondamentale supporto scientifico e grazie al quale ho scoperto il mondo della ricerca.

Vorrei ringraziare la mia collega nonché amica Ing. M. E. Lombardo per essere stata al mio fianco durante tutto il percorso di studi supportandomi in ogni situazione. Un grazie va anche alla mia collega E. Capuana con la quale è da poco nato un bel rapporto.

Vorrei ringraziare anche il Prof. G. Gherzi e i suoi collaboratori che sono stati fondamentali per la mia crescita professionale all'interno di un'azienda.

Vorrei esprimere la mia gratitudine al prof. Aldo R. Boccaccini e prof. Gundula Schulze-Tanzil per avermi accolta nel loro ambiente di lavoro altamente stimolante durante il mio periodo in Germania.

Un grazie anche a tutti gli studenti che hanno condiviso con me parte del mio PhD: Rossella Di Falco, Biagio Gullo, Aurelio Civilleri, Alessandro Trattato, Rossella Davi.

Infine volevo fare un grandissimo ringraziamento alla mia famiglia nonché il mio pilastro, che ha sempre creduto in me e supportato nelle difficoltà della vita.

Un ultimo grazie va a Vittorio Mistretta, il mio compagno di avventure. Infinitamente grazie per il coraggio, la forza trasmessa nei momenti difficili e per aver sempre creduto in me, ma soprattutto per avermi dato lo spunto di andare sempre avanti. GRAZIE!

Publications

Some ideas and figures have appeared previously in the following publications:

ISI Publications

1. I. Vitrano, F. Carfi Pavia, G. Conoscenti, M.E. Lombardo, V. La Carrubba, V. Brucato. Micro X-ray Computed Tomography: Evaluation of Hydroxyapatite distribution in a Poly-L-Lactic Acid (PLLA) scaffold via Thermally Induced Phase Separation (TIPS). Chemical Engineering transaction, 64:193-198, 2018.
2. M.E. Lombardo, F. Carfi Pavia, I. Vitrano, G. Gherzi, V. Brucato, V. La Carrubba. PLLA scaffolds with controlled architecture as potential microenvironment for in vitro tumor model. Tissue and cell, 58:33-41, 2019.
3. F. Carfi Pavia, M.A. Di Bella, V. Brucato, V. Blanda, F. Zummo, I. Vitrano, C.M. Di Liegro, G. Gherzi, I. Di Liegro, G. Schiera. A 3D scaffold of PLLA induces morphological differentiation and migration of primary astrocytes and promotes production of extracellular vesicles. International Journal of Molecular Medicine, 20:1288-1296, 2019.
4. F. Palumbo, F. Bongiovi, F. Carfi Pavia, I. Vitrano, V. La Carrubba, G. Pitarresi, V. Brucato, G. Giammona. Blend scaffolds with polyaspartamide/polyester structure fabricated via TIPS and their RGDC functionalization to promote osteoblast adhesion and proliferation. Journal of Biomedical Materials Research: Part A, 2019.

5. A. De Luca, I. Vitrano, L. Raimondi, V. Carina, V. Costa, D. Bellavia, G. Conoscenti, R. Di Falco, F. Carfi Pavia, V. La Carrubba, V. Brucato, G. Giavaresi. Improvement of osteogenic differentiation of human mesenchymal stem cells on composite PLLA/HA scaffolds for bone defect repair. *Journal of Bioscience and Bioengineering*, 2019.
6. I. Vitrano, F. Carfi Pavia, M.E. Elena, V. La Carrubba, V. Brucato. Micro X-ray Computed Tomography as technique for analysing structural properties of composite scaffolds. *Composites science and technology*. Submitted.
7. F. Lopresti, F. Carfi Pavia, I. Vitrano, M. Kersaudy- Kerhoas, V. Brucato, V. La Carrubba. Effect of hydroxyapatite concentration and size on morpho-mechanical properties of PLA-based randomly oriented and aligned electrospun nanofibrous mats. *Journal of the Mechanical Behaviour of Biomedical Materials*, 10.1016/j.jmbbm.2019.103449, 2019

International conference

1. I. Vitrano, F. Carfi Pavia, V. Brucato, C. Goegele, A.R. Boccaccini, V. La Carrubba, G. Schulze-Tanzil. Tubular composite scaffolds produced via Diffusion Induced Phase Separation (DIPS) as a shaping strategy for anterior cruciate ligaments reconstruction. *Paracelsus Science Get Together, Salzburg (Austria)*, 29th June 2018.
2. A. Civilleri, C. Goegele, S. Schwarz, K. Schäfer-Eckart, I. Vitrano, F. Carfi Pavia, V. La Carrubba, G. Schulze-Tanzil. Biological characterization of Poly-L-lactic acid/Hydroxyapatite/Bioglass composite scaffolds made by Thermally Induced Phase Separation (TIPS) hosting human Mesenchymal Stem Cells. *Paracelsus Science Get Together, Salzburg (Austria)*, 29th June 2018.

3. I. Vitrano, F. Carfi Pavia, A.R. Boccaccini, V. La Carrubba, V. Brucato. In vitro bioactivity study of composite scaffold prepared via thermally induced phase separation. Eurofillers Polymerblends. Palermo (IT), 23-26 April 2019.
4. I. Vitrano, F. Carfi Pavia, A.R. Boccaccini, V. La Carrubba, V. Brucato. BIOGLASS® pre-treatment improves its integration in polymeric scaffolds. Materials in the next decade, Favignana (Italy), September 2019.
5. F. Lopresti, I. Vitrano, F. Carfi Pavia, V. Brucato, V. La Carrubba. Bilayer tubular scaffold produced by diffusion induced phase separation and electrospinning. Workshop Materials in the next decade, Favignana (TP), 18-20 September 2019
6. I. Vitrano, F. Carfi Pavia, A.R. Boccaccini, V. La Carrubba, V. Brucato. Bioglass® pre-treatment improves its integration in polymeric scaffolds. Workshop Materials in the next decade, Favignana (TP), 18-20 September 2019
7. Microscopic evidence of the primary astrocytes' morphological differentiation and migration inside porous Poly-L-lactic acid 3D-scaffolds. 14 th Multinational Congress on microscopy (MCM2019), Belgrade (Serbia), 15-20 September 2019

National conference

1. M.E. Lombardo, I. Vitrano, F. Carfi Pavia, G. Conoscenti, V. Brucato, V. La Carrubba. Double Flow Perfusion Bioreactor for dynamic 3D cell cultures and In Vitro Test of Drug Delivery. GRICU PhD National School. Palermo (IT), 2017.
2. I. Vitrano, F. Carfi Pavia, A.R. Boccaccini, V. La Carrubba, V. Brucato. BIOGLASS® integration in Poly-L-Lactic-Acid scaffolds produced via Thermally Induced Phase Separation (TIPS). GRICU National conference. Palermo (IT), 30 Giugno - 3 Luglio 2019.

List of figures

Figure 1.1 A) Distribution of the different tissues studied for in vitro regeneration according to the terms found in article title, abstract and keywords. The “Others” section includes (from the most referenced to the least) lung, kidney, tooth, bladder, cornea, pancreas, ear, aortic valve, thyroid, and sphincter. B) Distribution of the different types of scaffolds in the most recent articles, from Baudequin et al., 201833

Figure 1.2 Tissue engineering process. Autologous cells of interest are extracted and isolated. After expansion preculture, they are seeded on a scaffold in specific environmental conditions (biological, chemical, and/or mechanical signals). The complex (cells + scaffold) is then implanted after maturation, adapted from Baudequin et al.,201834

Figure 1.3 Pie chart showing the percentages of the main bone tissue repair applications related to the global market of bone grafts and substitutes, adapted from Market Research Report, June 201835

Figure 1.4 (A) Published articles on BTE since mid-1980s on PubMed; (B) Partition of the BTE related publications of 2011 into different focuses, adapted from Amini et al., 2012.....36

Figure 1.5 Illustration of hierarchical structure of bone tissue in three levels: macrostructural, microstructural and nanostructural. Microstructure consists of Haversian systems (circles in cross-section and microscopic view) with osteonic canals and lamellae, and at the nanoscale, there are collagen fibers composed of bundles of mineralized collagen fibrils, from Sadat-Shojai et al. 201338

Figure 1.6 (A, B) Schematic structure and histologic transversal section of cortical bone; (C, D) Schematic structure and histologic transversal section of cancellous bone, adapted from Meyer,2006).	40
Figure 1.7 Illustration of a typical fracture healing process, biological events, and cellular activities at different phases, from Wang et al.,2017	43
Figure 1.8 Illustration of architecture of cartilage tissue, from Liu et al.,2017.....	44
Figure 1.9 A) ACL structure and histological section in normal condition; B) tendon/ligament architecture hierarchically organized into structural units, adapted from Santos et al.,2017 and Atesok et al., 2016	46
Figure 1.10 (A) Composite hip implant; (B) Polyether-ether-ketone (PEEK) cranial device; (C) Surgical bone grafting procedure from iliac crest; (D) Cortical bone graft	50
Figure 1.11 Comparison between traditional and tissue engineering approach in anterior cruciate ligaments diseases, from Nau et al., 2015.....	51
Figure 1.12 Elastic modulus versus compressive strength values of various BTE biomaterial classes compared to human bone, from Amini et al., 2012.....	57
Figure 1.13 Chemical structure of some natural polymers.....	58
Figure 1.14 Chemical structure of some synthetic polymers	62
Figure 1.15 Some of the conventional scaffold fabrication techniques; (A) Solvent casting/particulate leaching; (B) Gas foaming; (C) Emulsion freeze-drying; (D) Electrospinning, adapted from Sampath et al,2016	70
Figure 1.16 A) 3D printing; B) Fused deposition model for the fabrication of scaffolds	71

Figure 2.1 Solution's free enthalpy diagram as a function of the composition at temperature T.	79
Figure 2.2 Free enthalpy/volume fraction curves for different combinations of N_1 and N_2 : (a) $N_1/N_2 = 1/1$; (b) $N_1/N_2 = 1/1000$; (c) $N_1/N_2 = 100/1000$	82
Figure 2.3 Free energy of mixing as a function of composition for a binary mixture at different temperatures: here a thermodynamic instability can be noted between compositions ϕ_I and ϕ_{II} (adopted by Kim et al.,2017)	83
Figure 2.4 A) Correlation between ΔG_m and the temperature/composition phase diagram for a binary polymer-solvent system; B) Trend of the immiscibility region of a binary mixture in function of the molecular weight of the polymer: from curve "a" to curve "d" the molecular weight increases (adopted by Kim et al.,2017).	84
Figure 2.5 Thermally induced phase separation in polymer solutions: S-L phase separation and L-L phase separation with different final morphology, from Kim et al. (2017)	86
Figure 2.6 (A, B) Demixing process of a binary polymer solution promoted by a decreasing in the temperature; (C) Plots of ΔG_m and its derivatives versus ϕ	87
Figure 2.7 Three-dimensional region of immiscibility of the same ternary solution, derived from binodal curves taken at various temperatures, from Kim et al., 2017)	89
Figure 2.8 Ternary diagram containing a liquid-liquid demixing gap.	90
Figure 2.9 (A) Phase separation of a stable ternary solution induced by temperature decrease; (B) Phase separation promoted by the addition of nonsolvent in a stable binary solution.	91
Figure 2.10 Triangular diagram PLLA-dioxane-water	93

Figure 2.11 Morphology of membrane surfaces obtained with a double immersion, first in a dioxane/water bath, then in pure water, and desiccated at room temperature, from Montesanto et al., 2015	95
Figure 2.12 Three main routes for obtaining high molecular weight poly(lactic acid) PLA, from Sampath et al., 2016.....	97
Figure 2.13 Preparation of PDLA and PLLA from respective lactides, from Agrawal et al., 2003	97
Figure 2.14 Degradation reaction mechanism of PLLA by hydrolytic cleavage	98
Figure 2.15 Chemical structure of dioxane and its isomers	100
Figure 2.16 Crystallographic structure of hydroxyapatite.....	103
Figure 2.17 Property changes of bioglass materials about bone-bonding, from Hench, 2006	107
Figure 3.1 Schematic set-up of micro-CT analysis from the acquisition to 3D reconstruction.	112
Figure 3.2 (A) Cylindric scaffolds as prepared by TIPS; (B) VOIs for quantitative analysis: Three regions of 300 layers, top, middle, bottom.....	116
Figure 4.1 Steps of scaffold preparation via TIPS: I) Preparation of ternary solution and adding of HA (filler); II) thermal path to obtained scaffold via TIPS.....	127
Figure 4.2: Images of cross section reconstructed (PS1) of different type of scaffolds: A) neat PLLA; B) PLLA/HA 90/10 wt/wt; C) PLLA/HA 80/20 wt/wt; D) PLLA/HA 50/50 wt/wt	130
Figure 4.3 3D reconstruction of composite scaffold PLLA/HA 90/10 wt/wt: A) image pixel size of 3.75 μm (PS1); B) image pixel size of 1.25 μm (PS2)	131

Figure 4.4 Threshold range values with related binarized images for: A) interconnected pores (0-30); B) PLLA polymer mesh of scaffold (30-160); C) HA particles (160-254). Example of scaffold PLLA/HA 90/10 wt/wt	132
Figure 4.5 SEM micrographs of (A) neat PLLA scaffold; (B) 90/10 wt/wt PLLA/HA scaffold; (C) 80/20 wt/wt PLLA/HA scaffold; (D) 50/50 wt/wt PLLA/HA scaffold.	134
Figure 4.6 HA quantitative analysis by micro-CT for all composite scaffolds in each VOIs. HA values (%v/v) are reported as a function of nominal HA (%wt).	137
Figure 4.7 HA weight percentage determined by thermal gravimetric analysis (blue rhombus), micro-CT_PS1 (red x) and micro-CT_PS2 (green triangle).	137
Figure 4.8 (A) HA particles size distribution in composite scaffolds with different HA weight percentages: 10 %wt (orange), 20 %wt (green) and 50 %wt (blue); (B) HA particles size [μm]obtained by granulometry analysis.	138
Figure 4.9 Percentage relative cell viability of MC3T3-E1 cells cultured in two different cell culture medium: control and osteo-inductive medium, respectively. Effect of different cell culture medium was investigated. Error bars represent means $\pm$ SD for n = 3. Asterisks denoted a significative difference between two groups, ** p<0.01.....	143
Figure 4.10 Percentage change in the ratio of cell ALP activities of MC3T3-E1 cultured in osteo-inductive/control medium.	143
Figure 4.11 Comparison between particle size distribution of HA before (red line) and after (blue line) grinding treatment	148
Figure 4.12 SEM micrographs of PLLA-HA scaffolds produced via TIPS keeping the demixing temperature at 30 °C for 30 min: (A) weight ratio of 95/5, B) weight ratio of 93/7.	148

Figure 4.13 μ CT reconstruction of PLLA-HA 7 %wt scaffold. (A) 3D volume visualization by CTVox software of whole scaffold, (B) HA distribution inside this volume. Polymer is highlighted in grey and particles in red	149
Figure 4.14 (A) Cell viability percentage of MC3T3-E1 cells cultured on scaffold in PLLA-HA 5%wt and 7% wt for up to 49 days; (B) ALP activity of cells on PLLA-HA 7 %wt scaffold at 7, 14, 21 and 27 days. Effect of HA concentrations was investigated. Error bars represent means $\pm$ SD for n = 3. Asterisks denote a significant difference compared with the reference, * P<0.05, ** P<0.001.....	152
Figure 4.15 MC3T3-E1 cells visualization and quantification by fluorescence microscope on PLLA-HA 7% wt scaffolds after 1, 7, 14, 21, 28 and 49 days of culture. DAPI-stained nucleus in blue. Scale bars 5 μ m.	153
Figure 4.16 Steps of hydroxyapatite preparation via wet chemical method, from Sadat-Shojai et al. 2013	154
Figure 4.17 SEM micrographs, dried hydroxyapatite at 70°C	156
Figure 4.18 EDX spectrum of HA produced via wet chemical method.....	157
Figure 4.19 : XRD diffraction pattern: A) HA CamCeram® III, B) HA dried via wet chemical method	158
Figure 4.20 Scaffold 5%wt HA 30°C 30min. Two different fillers were used: A) HA _{sg} via sol-gel technique; B) HA _{cm} via wet chemical method.....	160
Figure 4.21 MC3T3-E1 cell morphology before seeding.....	161

Figure 4.22 Cell viability percentage of MC3T3-E1 cells cultured on scaffold in PLLA/HA ratio 95/5 %wt for up to 10 days. Effect of two types of HA particles was investigated. Pure PLLA scaffolds were used as control. Asterisks denote a significant difference, * $P<0.05$, ** $P<0.001$.	163
Figure 4.23 MC3T3-E1 cells visualization and quantification by fluorescence microscope on (A) pure PLLA, (B) PLLA/HA _{sg} (C) PLLA/HA _{cm} scaffolds after 5 days of culture. DAPI-stained nucleus in blue. Scale bars 50 μm .	163
Figure 5.1 A) SEM image of BG13-93; B) Atomic % concentration measured on the BG surface as received and after pre-treatment in dioxane-water 87/13 %wt for 1,7 days. O was removed from the calculations.	171
Figure 5.2 Comparison between the morphologies of scaffold PLLA-BG45S5pre-treated and PLLA-BG1393pre-treated produced via TIPS 30°C 30min in aluminium holder sample.	172
Figure 5.3 SEM micrographs of PLLA/BG scaffold obtained via TIPS changing to sample-holder, demixing temperature and time.	173
Figure 5.4 A) Stress-strain curves of PLLA, PLLA/5%BG13-93 and PLLA/5%BG13-93pret; B) Compressive Young's modulus, ** $p<0.01$.	174
Figure 5.5 SEM micrographs of BG13-93 pre-treated before (A) and after 7 days in SBF (B)	175
Figure 5.6 FTIR spectra (A) and XRD pattern (B) of BG13-93 powders as received, 3 days and 7 days after immersion in SBF. In red powder pre-treated in dioxane/water solution, in blue non-pre-treated powders	177
Figure 5.7 Schematic representation of Diffusion Induced Phase Separation (DIPS) process	180

Figure 5.8 Schematic representation of the experimental set-up for the cell biology study, from Balasubramanian et al. 2017.....	183
Figure 5.9 SEM micrographs of PLLA/BG 13-93 membrane: (A) external surface, (B) inner surface.....	186
Figure 5.10 TGA analysis: Mass loss (%) vs Temperature (°C) of PLLA/BG 13-93 membrane.	186
Figure 5.11 Three dimensional reconstruction of membrane PLLA/BG-13-93 with 10% filler by CTVox: (A) PLLA sheet (grey) BG particles (red), (B) distribution of BG particles, (C) membrane thickness, scale bar 250µm.	187
Figure 5.12 FTIR and XRD spectra of pure PLLA and PLLA/BGs membrane: (left) before and (right) after 14 days of soaking in SBF. The peaks indicated in this figure are discussed in detail in the text.	189
Figure 5.13 SEM micrographs of the surface of composite scaffolds after soaking in SBF for 14 days at different magnifications. (A) PLLA/BG45S5, B) PLLA/BG 13-93, (C) PLLA/BG 13-93B3.....	191
Figure 5.14 Relative viability of ST2 cells cultured with dissolution products of: a) BG powders; b) PLLA/BGs 10% wt membranes at different dilutions. Asterisks denote a significant difference compared with the reference, ** P<0.001.	193
Figure 5.15 Comparison between light microscopy images of H&E-stained ST2 cells cultured with 1% and 0.1% dilution of different BG powders and composites membranes.	195

Figure 5.16 VEGF release from ST2 cells treated with different dilutions of IDPs of: A) BG powders; B) BGs/PLLA 10% wt membranes at different dilutions. Asterisks denote a significant difference compared with the reference, * $P < 0.01$, ** $P < 0.001$	197
Figure 6.1 SEM micrographs of complete set of scaffolds as prepared via TIPS	203
Figure 6.2 Porosity evaluation of composite scaffolds PLLA-HA, PLLA-HA-BG 90/10, PLLA-HA-BG 80/20. Scaffold in pure PLLA was used as control.	204
Figure 6.3 Thermal properties of PLLA, PLLA/HA, PLLA/HA/BG 90/10 and PLLA/HA/BG 80/20 scaffolds. (A) crystallinity (%); (B) melting temperature ($^{\circ}\text{C}$).	205
Figure 6.4 Young's modulus of different scaffold. Significant difference between each composite with the neat PLLA (*) $p < 0.05$, (**) $p < 0.001$	206
Figure 6.5 SEM micrographs of all samples after treatment in SBF for 28 days.....	208
Figure 6.6 FTIR spectra of all samples, before and after treatment in SBF for 28 days	209
Figure 6.7 XRD patter of all samples, before and after treatment in SBF for 28 days	210
Figure 7.1 (A) Tubular scaffold 2 cm long for cell culture tests. (B) Lapine ACL fibroblasts monolayer before the seeding.....	214
Figure 7.2 (A) Schematic representation of hanging drop methods; (B) All spheroids culture in drop in a petri dish. The drops were prepared suspending 50,000 cells/ 50 μl of medium, adapted from Hoarau-Véchet et al. 2018.	217
Figure 7.3 SEM micrographs of tubular scaffolds prepared with an extraction speed of 16 cm/min at 45°C . (A-B) Internal surface of pure PLLA and PLLA/BG13-93, respectively; (C-E)	

cross section of pure PLLA scaffold with diameters of 1.2 mm and 2 mm;(D-F) wall thickness of pure PLLA scaffold with diameter of 1.2 mm and 2 mm, respectively.	222
Figure 7.4 Hematoxylin/eosin staining of 7 μ m thick sections of 1.2 mm PLLA. A) wall thickness; (B) cross section.	222
Figure 7.5 3D Reconstruction of 2mm PLLA/BG13-93 by CTVox software. (A) Cross section; (B) external surface; (C) inner surface. Polymer (green) and bioglass (red).....	223
Figure 7.6 Comparison between pores size [μ m] in the inner surface of pure PLLA and PLLA/BG13-93 tubular scaffold. Values are reported as mean value $\pm$ SD (n=10). Asterisks denote a significant difference between two groups (** P<0.01).	224
Figure 7.7 Cross section of all scaffolds stained with live/dead assay after 1, 7, 14 days of culture. Alive cells are visible in green colour, whereas dead cells are the red dots. Magnification: 50x.	229
Figure 7.8 Cross section of all scaffolds stained with live/dead assay after 1 and 14 days of culture. Alive cells are visible in green colour, whereas dead cells are the red dots. Pictures at two magnifications are shown at 14 days.	230
Figure 7.9 Spheroids of ACL fibroblast. (A) Live/dead staining before seeding; (B) control in plate after 7 days from the seeding; (C) Vimentin immunolabeling (red) and DAPI (blue) after 7 days (D) H&E staining after 7 days of culture in a scaffold.	231
Figure 7.10 Cross section of all scaffolds stained with live/dead assay after 1 and 14 days of culture (Protocol C). Pictures at two magnifications are shown at 14 days.	232
Figure 7.11 Longitudinal section of PLLA-BG13-93 d=1.2 mm scaffolds stained with live/dead assay after 1 day (A-C-E) for protocol A, B and C, respectively. (B-D-F) cells at 14 days	

of culture following protocol A, B, C respectively. Alive cells are visible in green colour, whereas dead cells are the red dots. Magnification: 100x233

Figure 7.12 Day 7 hematoxylin/eosin staining assay of sections of PLLA d=1.2 mm scaffolds seeded with: (A-C) cell suspension according to protocol A; (B-D) fibrin gel mediated seeding (protocol B). Magnification: 100x, 200x.....235

Figure 7.13 Hematoxylin/eosin staining of PLLA-BG D=2 mm scaffolds seeded with spheroids (protocol C) after 1 (A-C) and 14 days (B-D). Magnification: 100x, 200x.235

Figure 7.14: Immunolabelings of PLLA/BG13-93 scaffold seeded following protocol A (cell suspension). Cell nuclei are stained in blue and vimentin in red. (A-C) Cross and longitudinal section of a small scaffold, (B-D) cross and longitudinal section of a big scaffold.237

Figure 7.15 : Immunolabelings of 1.2 mm pure PLLA-BG13-93 scaffold with fibrin gel 7 days after seeding: cell nuclei (blue) and vimentin (red). Magnification 100x.237

Figure 7.16: Immunolabelings of 1.2 mm pure PLLA-BG13-93 scaffold with spheroids 7 days after seeding: (A) cell nuclei and vimentin; (B) collagen type I (green) and vimentin. Magnification 50x.238

Figure 7.17 Results of the CyQuant proliferation assay in terms of cell numbers present in each scaffold after 1 day of culture. Asterisks denote a significant difference compared with the protocol A, * P<0.05, ** P<0.001.240

Figure 7.18 Results of the CyQuant proliferation assay in terms of cell numbers present in each scaffold for 1.2 mm PLLA-BG after 1 and 7 day of culture. Asterisks denote a significant difference compared with the protocol A, * P<0.05, ** P<0.001.241

Figure 7.19 Representative phase contrast photomicrographs of L929 and lapine ACL cell cultures, before incubating with the medium containing BG ionic dissolution products.....	244
Figure 7.20 Changes in pH caused by ions release of BG45S5 and BG13-93. pH values of solutions at different concentrations. Fresh medium was used as control. The results represent average value of measurements (n=3) with error bars giving SD values. Statistical significance levels were set at $p < 0.05$ (*), $p < 0.01$ (**).....	245
Figure 7.21 Live/dead staining of ACL fibroblast in culture with IDPs of BG45S5 and BG13-93 at different concentration.....	246
Figure 7.22 Hematoxylin/Eosin staining of ACL fibroblast in culture with IDPs of BG45S5 and BG13-93 at different concentration	247
Figure 7.23 Live/dead staining of L929 in culture with IDPs of BG45S5 and BG13-93 at different concentration.....	247
Figure 7.24 Hematoxylin/Eosin staining of L929 in culture with IDPs of BG45S5 and BG13-93 at different concentration.....	248
Figure 7.25 the percentage of colonized area of living cells respect the total area of the live/dead picture.	248
Figure 7.26 Relative viability of LKB, L929 cells IDPs of different BG composition and dilutions. Asterisks denote significant difference compared with the reference, * $p < 0.05$; ** < 0.001	250
Figure 7.27 SEM micrographs of A) internal layer of DIPS-made tube; B) electrospun mats; (C, D) bilayer tubular scaffold.....	253

List of tables

Table 1.1 Advantages and Disadvantages of Conventional Processes for Fabricating Scaffolds	66
Table 2.1 Composition of 45S5, 13-93 and 13-93B3 glasses (in wt%) used in this research project	106
Table 3.1 Ions concentrations of SBFs and human blood plasma	121
Table 4.1 Thresholding values used for images binarization depending on the typology of quantitative analysis	131
Table 4.2 Porosity of composite scaffolds at different PLLA/HA weight ratios by different methods. Mean value was evaluated between sections of Top, Middle and Bottom and then standard deviation (SD) was calculated.....	133
Table 4.3 Average pore size [μm] of scaffold with 0, 10, 20, 50 %wt HA. Comparison of values obtained via different techniques.	135
Table 4.4 Comparison between theoretical and experimental molar ratio of Hydroxyapatite	157
Table 7.1 Mechanical properties of pure PLLA and PLLA-BG13-93 tubular scaffold with different diameters.	226
Table 7.2 Percentage of attached cells in all scaffolds, after 24 h of dynamic seeding	227
Table 7.3 Mechanical properties of tubular scaffold produced via DIPS, electrospun fibers and by-layer scaffold DIPS/ES.....	253

Table of contents

Abstract	1
Sommario	5
Acknowledgements	9
Publications	10
List of figures	13
List of tables	25
1 Introduction	32
<i>1.1 Overview on Bone Tissue Engineering</i>	<i>32</i>
<i>1.2 Anatomy and physiology of bone tissues</i>	<i>37</i>
1.2.1 Bone	37
1.2.2 Cartilage	43
1.2.3 Anterior cruciate ligaments	45
<i>1.3 Traditional strategies for the treatment of tissue disease</i>	<i>48</i>
<i>1.4 Scaffold applications for tissue repair</i>	<i>52</i>

1.5	<i>Suitable materials for BTE scaffolds</i>	56
1.5.1	Natural polymers	58
1.5.2	Synthetic polymers	61
1.6	<i>Scaffold fabrication technique</i>	65
1.7	<i>Cell line for BTE and cells-scaffold interactions</i>	72
2	Aim of this work and strategies for preparing composite scaffold	74
2.1	<i>Aim of research work</i>	74
2.2	<i>Thermally Induced Phase Separation (TIPS)</i>	77
2.3	<i>Diffusion Induced Phase Separation (DIPS)</i>	92
2.4	<i>Polymeric solution</i>	95
2.4.1	PLLA	96
2.4.2	Dioxane	99
2.5	<i>Solution with filler</i>	100
2.5.1	Hydroxyapatite	102
2.5.2	Bioactive glass	104
3	Composite scaffolds characterization	110
3.1	<i>Materials characterization</i>	111
3.1.1	Micro-computed tomography (μ CT): Reconstruction and analysis	111

3.1.2 Scanning electron microscope (SEM)	117
3.1.3 Porosity evaluation	118
3.1.4 Thermal gravimetric analysis (TGA)	118
3.1.5 Mechanical testing	119
3.1.6 XRD analysis	119
3.1.7 FTIR analysis	120
3.1.8 In vitro bioactivity study	121
3.2 <i>Biological characterization</i>	122
3.2.1 Live/dead cell viability assay	122
3.2.2 Haematoxylin/Eosin histologic assay	123
3.2.3 Vitality test	123
3.2.4 <i>Statistical analysis</i>	124
4 Composite scaffold PLLA-HA	125
4.1 <i>PLLA-HA scaffolds</i>	125
4.1.1 Scaffold preparation	126
4.1.2 Scaffold characterisation	127
4.1.3 Results	132
4.2 <i>Osteoblast differentiation in 2D culture</i>	140
4.2.1 In vitro cell culture	140
4.2.2 Cell proliferation and differentiation	141

4.2.3 Results	142
4.3 <i>Osteoblasts in composite PLLA/HA scaffolds</i>	144
4.3.1 Scaffolds preparation and characterization.....	144
4.3.2 In vitro cell culture	145
4.3.4 Cell viability assay.....	146
4.3.5 Fluorescence microscope.....	146
4.3.6 Alkaline phosphatase activity	146
4.3.7 Results	147
4.4 <i>HA synthesis and characterization</i>	154
4.4.1 CompositePLLA/(insitu)HAScaffolds	159
4.4.2 Cells culture and viability assay	160
4.4.3 Results	161
4.5 <i>Outline</i>	163
5 Composite scaffold PLLA-BG	166
5.1 <i>Composite scaffolds produced via TIPS</i>	167
5.1.1 Bioglass pre-treatment	167
5.1.2 Scaffold preparation	167
5.1.3 Bioglass powders and scaffold characterisation.....	168
5.1.4 Results	169
5.2 <i>Composite membrane produced via DIPS</i>	178

5.2.1 Membrane preparation.....	179
5.2.2 Membrane characterisation.....	180
5.2.3 In vitro bioactivity study.....	181
5.2.4 In vitro study of angiogenic potential.....	181
5.2.5 Results	185
5.3 <i>Outline</i>	198
6 Composite scaffolds PLLA-HA-BG	200
6.1 <i>Scaffold preparation</i>	200
6.2 <i>Scaffold characterization</i>	201
6.2.1 Differential scanning calorimetry analysis	201
6.2.2 In vitro bioactivity test.....	202
6.5 <i>Results</i>	202
7 Scaffold for Ligaments regeneration.....	211
7.1 <i>Composite tubular scaffold</i>	212
7.1.1 Scaffold preparation	212
7.1.2 Scaffold characterisation	213
7.1.3 In vitro cell culture	214
7.1.4 Cell seeding protocols	214
7.1.5 Cell viability and morphology	218

7.1.6 CyQuant proliferation assay	219
7.1.7 Results	220
7.2 <i>In vitro</i> bioglass cytotoxicity test.....	242
7.2.1 Cell culture	242
7.2.2 Preparation of CCM with BG dissolution products.....	242
7.2.3 Results	244
7.3 <i>Bilayer tubular scaffold</i>	250
7.3.1 Scaffold preparation	251
7.3.2 Scaffold characterization	251
7.3.1 Results	252
Conclusion	255
Future work	259
Reference	260

Chapter 1

1 Introduction

1.1 Overview on Bone Tissue Engineering

The bone is a living complex porous composite, constantly changing, tissue. It has unique properties of remodelling to adapt its microstructure to external mechanical stress (Mathieu et al. 2006). Despite the benefit that surgical techniques have brought, defects caused by traumatic injury, disease or tumour resection cannot be repaired physiologically. Bone tissue engineering (BTE) methodologies and techniques were greatly improved, providing a multidisciplinary approach for the replacement and regeneration of damaged or lost cartilage and bone tissue to overcome limitations of traditional autografts and allografts, such as risk of immune rejection, pathogen transfer and inflammation. The regeneration of tissues is mediated by specific biocompatible and biodegradable scaffolds, defined as biodegradable synthetic 3D devices emulating the extracellular matrix (ECM), whose major function is to provide a temporary support for tissue structures. Ideal scaffolds should promote cellular interactions and tissue development, and possess proper mechanical and physical properties (X. Liu and Ma 2004). New approach involves the development of in vitro rebuilt substitutes as alternatives to grafts and biomaterials used alone (Baudequin and Tabrizian 2018),

through combinations of biomaterials and biologics such as proteins, cells and growth factors inducing the bone regeneration (Boerckel et al. 2014).

In 2017, more than 40% of the literature studies was focused on analysis of bone and cartilage tissue for *in vitro* regeneration (Figure 1.1 A). Among all the materials, an enormous interest in bioresorbable materials especially for hydrogels and polymers (~65%) has been recently shown (Figure 1.1 B).

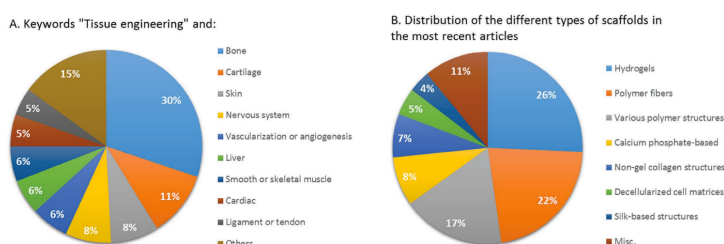


Figure 1.1 A) Distribution of the different tissues studied for in vitro regeneration according to the terms found in article title, abstract and keywords. The "Others" section includes (from the most referenced to the least) lung, kidney, tooth, bladder, cornea, pancreas, ear, aortic valve, thyroid, and sphincter. B) Distribution of the different types of scaffolds in the most recent articles, from Baudequin et al., 2018

The BTE is based on three major interplaying points: a scaffold, cells of interest and an appropriate environment (growth and differentiation factors, external stimuli such as mechanical solicitations). Each aspect must be optimized to obtain implantable tissue-engineered constructs, with an autologous process using the patient's own cells when possible (Figure 1.2).

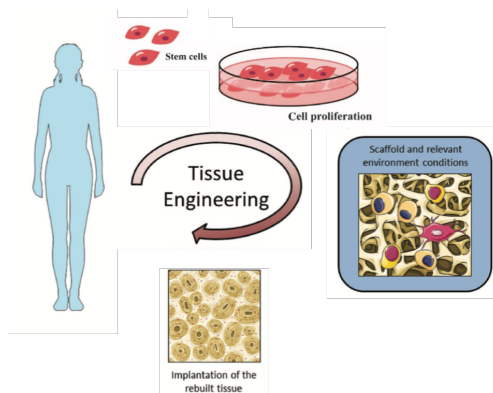


Figure 1.2 Tissue engineering process. Autologous cells of interest are extracted and isolated. After expansion preculture, they are seeded on a scaffold in specific environmental conditions (biological, chemical, and/or mechanical signals). The complex (cells + scaffold) is then implanted after maturation, adapted from Baudequin et al., 2018

In 2017, a very interesting value was recorded: the global bone grafts and substitutes market reached 2.69 billion USD. Moreover, a more impressive forecast affirms that the same market will be valued at about 3.912 billion USD in 2025 (Srivastava, 2018). According to the European Commission, the number of surgical procedures of cartilage substitution which are carried out every year in Europe is around 800,000, with a total cost of 1.5 billion USD. However, there is in addition other 2.63 billion USD attributed to an estimated number of failed implants (about 20%), which underwent a corrective surgery (Market Research Report, November 2017). Other major applications include craniomaxillofacial, dental, foot and ankle, joint reconstruction, long bone and spinal fusion (Figure 1.3).

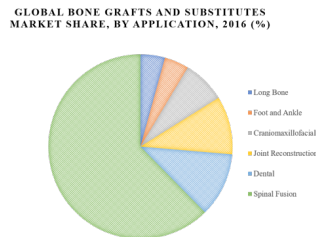


Figure 1.3 Pie chart showing the percentages of the main bone tissue repair applications related to the global market of bone grafts and substitutes, adapted from Market Research Report, June 2018

This progressive elevation is mostly due to the increasing obesity and ageing of the global population, which contribute to the raise of some bone-related disorders like osteoporosis and osteoarthritis. Another frequent disturb is the pseudarthrosis caused by impaired bone healing due to obesity and life style parameters such as smoking, but could also be congenitally (Market Research Report, June 2018). Besides, cartilage and subchondral bone damage can be caused by a variety of conditions, such as arthritis, trauma sports-related injuries and overuse. Recent studies have shown how bone, cartilage or anterior crucial ligaments injuries are increased in the last years. *Ghassemi et al. 2018* reported that incidence for all fractures among United States population in 2010 was 4017/100,000. *Liu et al. 2017* reported that 60% of patients examined by knee arthroscopy exhibit cartilage damage, and ~15% of people over 60 years old have some clinical symptoms of such damage. Also, anterior cruciate ligament injuries are among the most common athletic injuries in young population, as reported by *Singh 2018*. It is estimated that in the United States of America (USA), there are 200,000 ACL injuries,

half of which require reconstructive surgeries. The incidence estimates an increase in the athletes population, especially the value of 14/100,000 soccer players exposures per year; 14/100,000 skiers exposures per year between 1989 and 2004.

In this respect, BTE went through a massive advance over the last years. Attraction towards this field is attested by an ever-growing number of studies and reviews published on scientific research databases (Figure 1.4). It is an interdisciplinary area that has science, engineering and surgery concepts inside, and its final goal is to create functional tissues able to repair and regenerate bone injuries, in contrast with traditional bone repair strategies, aiming only to implant new spare parts (Kneser et al. 2002).

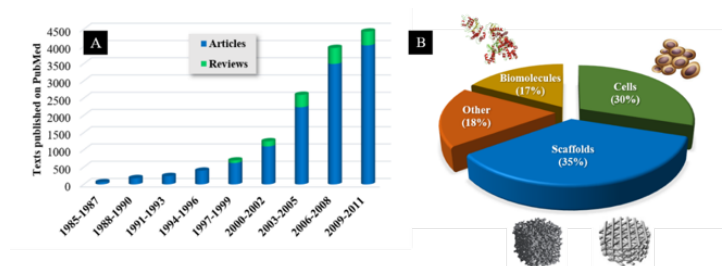


Figure 1.4 (A) Published articles on BTE since mid-1980s on PubMed; (B) Partition of the BTE related publications of 2011 into different focuses, adapted from Amini et al., 2012

The key players for a proper BTE product are:

- an adequate biocompatible scaffold that closely mimics the natural bone extracellular matrix;

- osteogenic cells to be fixed into the scaffold, in order to produce bone tissue within the artificial matrix;
- morphogenic signals, fundamental to induce and accelerate cell differentiation into the desired phenotype;
- wide vascularization throughout the structure, which assures nutrient supply to cells and waste products removal.

Thanks to this meaningful aspects, BTE approach can be recognised as a superior practice to promote bone reconstruction: in fact, the repair process may concurrently evolve with the patient's own tissue, until the regeneration is completed.

1.2 Anatomy and physiology of bone tissues

A crucial aspect in the BTE is understanding of the biology and structure of extracellular matrix of different bone tissues to realize successful tissue regeneration.

1.2.1 Bone

Bone is a highly vascularized biomineralized connective tissue with high mechanical strength and structural complexity. The skeleton can even be seen as a mineral source, considering its capacity to quickly mobilize mineral deposits, depending on metabolic demand. It hence plays an active part in the body's physiology (e.g. calcium homeostasis). Beyond that, it facilitates motion, supporting muscular contraction, and has

a protective function towards internal organs. As a highly vascularized tissue bone possesses the unique feature to heal and remodel without leaving any scar (Sommerfeldt and Rubin 2001).

Bone structure consists of a variety of cell types distributed within an extracellular matrix, composed of a mineral fraction of hydroxyapatite (65-70%) and an organic fraction (25-30%) containing collagen (collagen type I represents the dominant matrix protein in bone) and other proteins like proteoglycans and sialoproteins (Sikavitsas, Temenoff, and Mikos 2001; W. Wang and Yeung 2017). A distinct hierarchical structural organization at the macrostructural, microstructural, and nanostructural levels can be observed in natural bone tissue Figure 1.5.

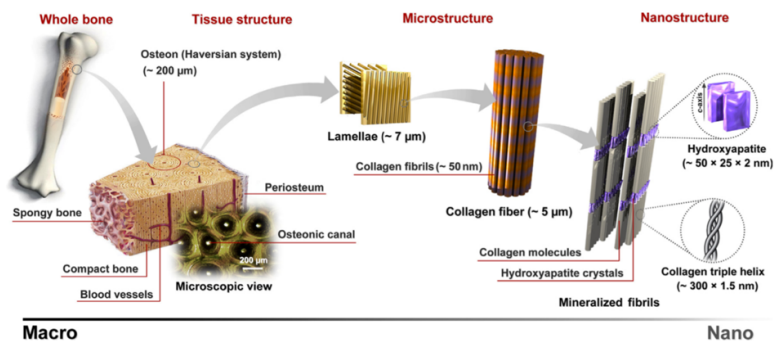


Figure 1.5 Illustration of hierarchical structure of bone tissue in three levels: macrostructural, microstructural and nanostructural. Microstructure consists of Haversian systems (circles in cross-section and microscopic view) with osteonic canals and lamellae, and at the nanoscale, there are collagen fibers composed of bundles of mineralized collagen fibrils, from Sadat-Shojai et al. 2013

At the macrostructure level, two distinct architectural forms can be observed, each of which assigns different structural and functional properties. The first arrangement is called cortical or compact bone (around 80% of the total skeleton), a highly dense bone, only 10% porous, surrounding the marrow space and conferring mechanical strength to bone (Figure 1.6 A, B). Cortical bone, in turn, is covered with the outer component of bone tissue, the periosteum, a thin layer containing essentially blood vessels and osteoblasts, whose duty is to get involved during appositional growth and bone repair processes. The second design, known as cancellous or trabecular bone (around 20% of the total skeleton), lies in the inner zone of some bones, has an irregular porous structure (estimated porosity in the range between 50% and 90%) with a framework interconnected trabeculae. The spongy-like form suited to provide accommodation and mechanical support for bone marrow interspersed inside, by transferring the loads through the bone (Figure 1.6 C, D). It can be found, protected by the cortical bone, in the metaphysis of long bones and in the vertebral bodies.

At the microstructure level, the elementary unit of the cortical bone is the osteon, a densely packed 20-30 collagen fibrils system organized in a concentric lamellar pattern, which surround the central canal and contain various blood vessels and nerves. The osteon hosts cells as osteoblasts and mature osteocytes, which cooperate in the generation and maintenance of extracellular matrix. Within the osteon, there is the haversian canal: inside it, nerves and blood vessels reside and are assembled in a three-dimensional network, starting from the centre of each osteon and crossing the cortical bone layer.

Finally, at the nanostructure level, there are large amounts of collagen fibers, which are composed of periodic collagen fibrils and gaps between the collagen molecules. Between collagen molecules, calcium phosphate crystals and non-collagenous organic proteins are embedded, which are the main components of the trabeculae and osteon units.

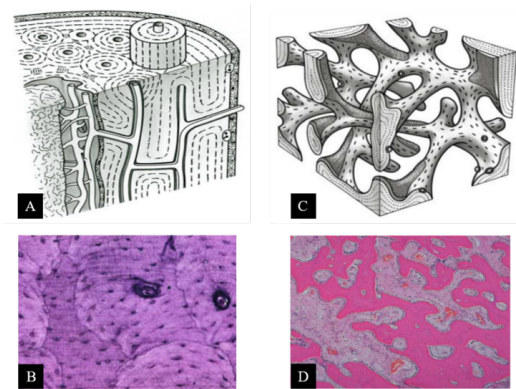


Figure 1.6 (A, B) Schematic structure and histologic transversal section of cortical bone; (C, D) Schematic structure and histologic transversal section of cancellous bone, adapted from Meyer, 2006).

The constant remodelling potential throughout human lifetime makes bone a highly dynamic tissue. The ability to synthesize new bone tissue belongs to osteoblasts, derived from the proliferation and differentiation of osteoprogenitor cells. Osteoblast phenotype permits cells to regulate matrix mineralization and collagen production thanks to the expression of functional proteins such as osteocalcin and alkaline phosphatase (ALP). In

its ultimate stage, osteoblast is transformed in a mature osteocyte: it loses some of the previous traits and acquires new specific characteristics, including the typical morphology. Osteocytes are in fact stellate or dendritic cells that not only produce, but also resorb bone matrix to a limited extent. Their network layout is crucial to properly adapt bone to external stimuli (i.e. bone development, turnover and repair) and to manage blood-calcium homeostasis. In contrast to cells introduced so far, osteoclasts are multinuclear cells originating from the fusion of mononuclear precursors, the hematopoietic stem cells, and have a macrophage-like differentiation route. Thereby, they are responsible of bone resorption activities implying the degradation of the mineral and organic bone matrix. This means dissolution of hydroxyapatite minerals and proteolytic cleavage of organic molecules, respectively (Meyer and Wiesmann 2006). When trauma or fractures damage bone tissue, the body responds by activating the regenerative process. The biological events and activities, as well as the cells involved in typical bone fracture healing at different phases are illustrated in (Figure 1.7). The healing process can be divided in three major steps: inflammation, repair and remodelling. In the first stage, signals released from the injured bone matrix activate the immune system, fundamental for an effective healing. Metabolic activity is suddenly activated with the increasing bone volume (in the first 5 days) by recruiting stem cells differentiation and retardation with chondrocyte apoptosis. Subsequently, the repair and remodelling processes start with the proliferation and migration to the defect site of the osteoprogenitors and osteocytes, coming primarily from the periosteum, but also from the adjacent cortical and cancellous bone. Mesenchymal stem cells also are associated with the formation of new bone: during

the repair process, they move from the surrounding muscle or from the marrow space to the affected zone. Once cells reached the critical area, they build a bony bridge to achieve the fracture healing (Figure 1.7); they previously synthesize a web of collagenous tissue, especially made up of type I, II and X collagen (5-10 days). Because a mineralized and mechanically efficient tissue is the final result of repair and regeneration processes, collagens thus delivered undergo a further mineralization mechanism; they stimulate indeed the microenvironment favouring $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ crystals nucleation. As a consequence, the extracellular matrix found in bone, if compared to other tissues, is highly mineralized and improves thereby the mechanical strength of the structure. Some studies demonstrate that despite collagen has the dominant role in orienting the mineralization step, if alone, it is not a direct nucleator of hydroxyapatite. Even three different non-collagenous proteins are of special relevance during bone formation: osteocalcin, osteonectin and bone sialoprotein. They come from the osteoblasts and are present at high concentrations in sites of bone remodelling, promoting cell attachment and regulating crystal growth by binding to hydroxyapatite. Nevertheless, the success of bone repair tissue strongly depends on the defect size: a bone defect can be spontaneously healed if it is below a critical size. Otherwise, it is not completely filled by new bone tissue, hypertrophic non-union occurs and the application of bone grafts is necessary. Another important parameter which determines the fate of new tissue formation is nutrition: the outcome of histogenesis is therefore truly influenced by diffusion and vascularization into the fracture site (W. Wang and Yeung 2017).

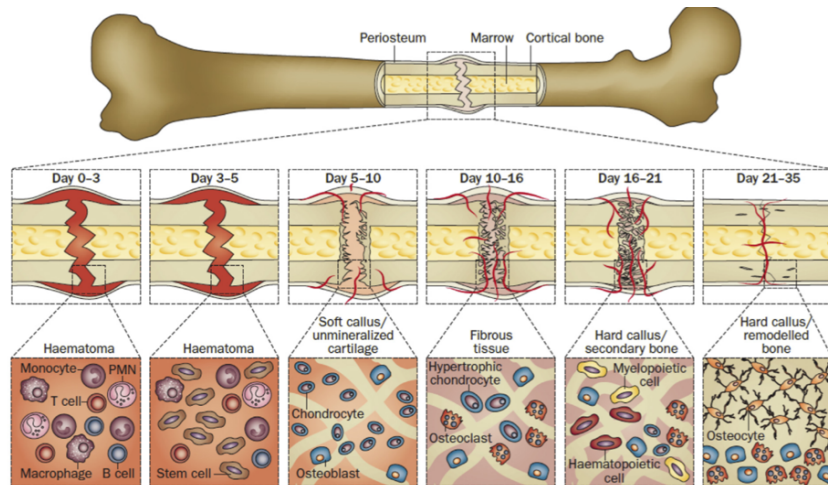


Figure 1.7 Illustration of a typical fracture healing process, biological events, and cellular activities at different phases, from Wang et al., 2017

1.2.2 Cartilage

In contrast to bone tissue, the cartilage is not vascularized connective tissue, with a structure rather simple. Cartilage is a fibers-reinforced composite material composed of chondrocytes surrounded by ECM composed of structural and functional proteins, glycoproteins, and glycosaminoglycans (Benders et al. 2013). Figure 1.8 shows composition and structure of cartilage where four different zones can be identified on the basis of collagen fibers alignment and proteoglycan composition (M. Liu et al. 2017).

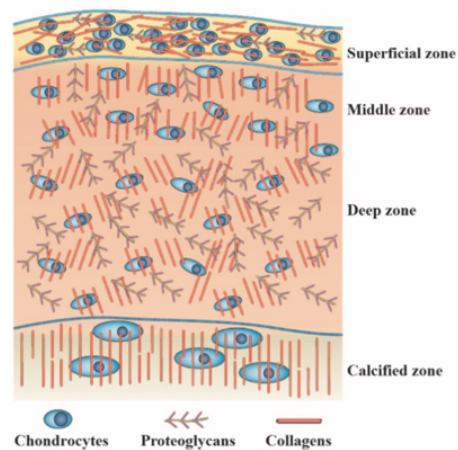


Figure 1.8 Illustration of architecture of cartilage tissue, from Liu et al.,2017

From the superficial zone to the deep zone, the proteoglycan content gradually increases. In the deep zone, there is the highest number of proteoglycans with collagen fibers arranged radially. Collagen fibers are aligned to the surface in the superficial zone, became unaligned and tangential to the cartilage surface in the middle zone and tend to organize in the calcified zone.

Owing to lack of blood vessels and of undifferentiated cells, the cartilage has limited capacity for repair, even minor lesion or injuries may lead to progressive damage with loss of function. The repair of cartilage depends on extent of the tissue distribution and ability of chondrocytes to sense changes in matrix composition and synthesize new

molecules. The chondrocyte can repair the tissue only in specific cases: the loss of proteoglycans does not exceed what the cells can rapidly produce, fibrillar collagen mesh remains intact, enough chondrocytes remain capable of regenerate the matrix damage. If the damage is too deep the health process is too slow and not effective, so it is necessary to intervene surgically (Meyer and Wiesmann 2006).

1.2.3 Anterior cruciate ligaments

The anterior cruciate ligament (ACL) is one of a pair of cruciate ligaments (the other being the posterior cruciate ligament) in the human knee. The two ligaments are also called cruciform ligaments, as they are arranged in a crossed formation and they control the back and forth motion of knee. The ACL is a key structure in the knee joint as it resists anterior tibial translation and rotational loads (Duthon et al. 2006). It runs diagonally in the middle of the knee, especially from the posterior medial aspect of the lateral femoral condyle in the intercondylar notch to the anterior aspect of the intercondylar eminence of the tibia (Figure 1.9 A), limits anterior movement of the tibia relative to the femur.

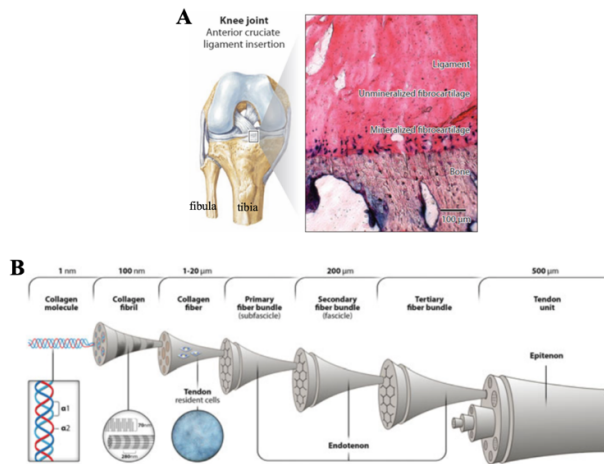


Figure 1.9 A) ACL structure and histological section in normal condition; B) tendon/ligament architecture hierarchically organized into structural units, adapted from Santos et al., 2017 and Atesok et al., 2016

The ACL is a band of dense connective tissue enveloped into the synovial membrane that connects the femur and the tibia. The cross-sectional shape of the ACL is “irregular” with values of 36 mm² and 44 from the femur to the tibia. The ACL consists of soft connective tissues (Zantop, Petersen, and Fu 2005). It is composed of multiple fascicles, each composed of collagen fibrils surrounded by connective tissue. Fascicles range from 250 μm to several millimeters, into 3–20 sub-fasciculi each of 100–250 μm in diameter. The tendon/ligament hierarchical structure (Figure 1.9 B) well-organized from the nano

to the microscale confers the anisotropic nature of tendons and ligaments and is responsible for the biomechanical properties of these tissues.

The matrix of the ACL consists of four different systems: collagen, glycosaminoglycans, glyco-conjugates and elastin. Major component, type I collagen fibrils are oriented parallel to the longitudinal axis of the ligament and responsible for the tensile strength of the ligament. Type II collagen is found in the fibrocartilaginous regions of the ACL, specifically the tibial and femoral sites of attachment. Type III collagen is located in the loose connective tissue that divides the type I collagen bundles. Fibronectin (a glyco-conjugate) is fundamental for intra- and extracellular matrix morphology, cellular adhesion, and cell migration. While, elastic components like mature elastic fibers permit the extreme distance changes during the motion.

Anterior cruciate ligament injuries do not heal due to its limited vascularization and surgical intervention is usually required. Most ACL tears are a result of a non-contact mechanism such as hyperextension of knee or a sudden change in a direction causing the knee to rotate inward. Others cause could be sudden stop, landing after a jump, or direct contact to the knee. About 50% of all injuries occur along with damage to other structures in the knee, such as articular cartilage, meniscus, or other ligaments (Anterior Cruciate Ligament Injuries-OrthoInfo, 2017). Injured ligaments are classified depending on the severity of the injury. Naturally, the type of treatment depends on several factors like the injuries grade, the age of patient.

1.3 Traditional strategies for the treatment of tissue disease

Bone

Nowadays, traditional methods of bone defect management are still of key significance in order to meet the clinical needs in the bone replacement and regeneration field. Among these, the most frequent current treatments are based on the use of biomaterials, autologous bone grafts and allografts.

Metals, like steel or titanium alloys, ceramics and polymers, are biomaterials commonly used for prosthetic devices (Figure 1.10 A, B), with a view to maintain skeletal function. Unfortunately, each of these materials has specific disadvantages: metals, for instance, poorly integrate with the surrounding tissue at the implantation site, regardless of bringing immediate mechanical support. Hence, a certain number of implants can fail afterwards, due to infection onset or to fatigue loading. Ceramics, on the other hand, display scarce mechanical properties, such as low tensile strength, considerable brittleness and they cannot be used in spaces where significant torsion, bending or shear stresses occurs (Yoshikawa et al. 2009). None of these “non-living”, non-resorbable biomaterials can compete with bone repair techniques performed by the use of autologous cells, which contain living cells and tissue inducing substances and are deemed to be the gold standard. Within this group, two distinct ways to repair bone defects exist: one relies on the improvement of the local host cell population, while the other involves the transplantation of grafted bone. The first procedure accelerates the healing process of bone lesions, but its performance depends on the conditions of the

tissue defect site: whenever there is the presence of wound infection and tissue necrosis or irritation, this protocol is unsatisfactory. Augmentation of the host cell population can be obtained through several pathways; one of these is, for example, the Guided Bone Regeneration (GBR). GBR exploits the action of a membrane as a barrier to separate bone tissue from the soft tissue ingrowth. The limit of GBR is to be ineffective towards geometrically complex or large defect sites (Pajoumshariati et al. 2018). Bone tissue regeneration could be finally stimulated from a biological viewpoint. Introduction of bone-related bioactive factors, among which Transforming Growth Factor beta (TGF β), Bone Morphogenic Proteins (BMPs) and Platelet-Derived Growth Factors (PDGFs) enhances bone cell reactions so that a faster and more targeted bone formation is accomplished (Kneser et al. 2002; De Witte et al. 2018).

The second approach is associated with a grafting operation of autologous bone tissue from the patient's donor site to the bone lesion (autograft) (Figure 1.10 C, D). One important reason for the preference of tissue grafts over the other bone reconstruction modes is that they contain own patient's living cells and, at the same time, tissue-inducing substances: these properties ensure to bone autografts a significant biological plasticity (W. Wang and Yeung 2017). In this case, some relevant problems are encountered, too: the majority of bone grafts are avascular and depends on diffusion, which is a very slow process; the bone resorption is unpredictable and bone grafts often are entirely resorbed by the body before osteogenesis is totally complete. The operating time required to harvest autografts is high, the number of autografts available from the donor are limited and, finally, they are susceptible to infections, pain and hematoma,

eliciting donor site morbidity. To overcome the problem of the limited availability, an alternative could be bone tissue extraction from somebody else's body; bone demineralized matrix represents an example. However, this second solution, called allograft, manifests some serious disadvantages: the rate of graft incorporation is lower than with the autografts and it introduces the risk of pathogen transmission and immune rejection from donor to host. Since each of these strategies possesses some disadvantages, an adequate bone replacement needed for fully recovery of the patients has yet to be found. BTE may solve some of these concerns through the development of bioengineered 3D matrices suitable to take the place of the current bone grafts for healing large bone defects.

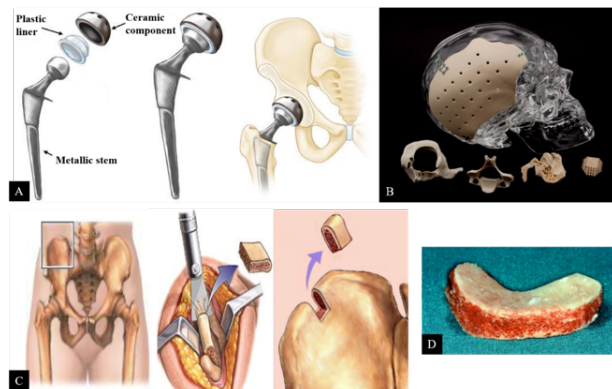


Figure 1.10 (A) Composite hip implant; (B) Polyether-ether-ketone (PEEK) cranial device; (C) Surgical bone grafting procedure from iliac crest; (D) Cortical bone graft

Anterior cruciate ligament

The treatment of ACL injuries involved different strategies, from non-operative care to several surgical procedures. In the 1970s, simple primary suturing was abandoned due to bad clinical results. Augmented ACL repair using natural as well as synthetic grafts leads to somewhat improved results. Synthetic grafts were popular in the 1980s but resulted in serious complications and bad clinical results. From the early 1990s onwards, ACL reconstruction with autograft or allograft material has become the method of choice for most surgeons (Figure 1.11). Among main disadvantages, transmissions of blood-borne diseases and the delayed biological incorporation were identified. Typically, structures that are injured or lost due to trauma and disease are the ideal candidates to be engineered (Nau and Teuschl 2015). Recently a new approach for the ACL regeneration is proposed by tissue engineering based on four components such as a structural scaffold, a cell source, biological modulators and mechanical modulator.

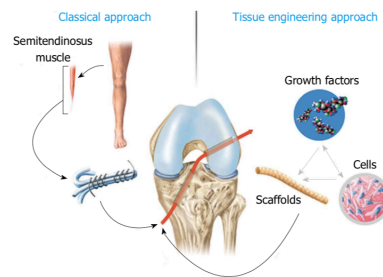


Figure 1.11 Comparison between traditional and tissue engineering approach in anterior cruciate ligaments diseases, from Nau et al., 2015

1.4 Scaffold applications for tissue repair

Cells are not the only component of a healthy and functional tissue: a matrix supplying a tissue specific environment and architecture to cells is necessary, too. In tissue engineering, 3D scaffolds are developed to mimic the extracellular matrix (ECM) and provide mechanical support thereby aiding in the formation of new tissue. In the particular case of BTE, the scaffold may actively participate to bone in-growth until the complete regeneration of the bony tissue by releasing growth or differentiation factors present in its structure. The advantages of these acellular scaffolds for bone tissue engineering include the ease of sterilization, longer shelf-lives and low potential for infection or immunogenicity (De Witte et al. 2018). Scaffold characteristics that can be modulated, improved or changed to make a scaffold suitable for BTE applications can be grouped into four main types: biological requirements, structural features, biomaterial composition and types of fabrication process. The first two features are defined and described below, while a small overview of materials adopted and fabrication process will be presented in the next paragraphs.

First of all, a scaffold should be *biocompatible* and not toxic, which means well integrated to the host tissue, supporting normal cellular activity, such as molecular signalling systems, without unleashing an immune response determined from local and systemic toxic effects to the host tissue. This quality is probably the main requirement for any bone scaffold (Williams 2008).

Also, scaffold's *bioresorbability* and *biodegradability* have to be considered: an optimal scaffold should degrade with time in proportion with the growth rate of the new tissue, creating new space for the further growth of the tissue itself. The best situation occurs when, concurrently, the regeneration process of the injured tissue is totally completed and the scaffold is totally degraded. The degradation kinetics of the scaffolds should vary based on the application in which they are used. Variation of mechanical strength during degradation is critical in load bearing implants, since the load should be gradually transferred to the regenerating tissue. In addition, even degradation products should be non-toxic and able to be excreted by the body (Roseti et al. 2017).

High *porosity* is another pivotal attribute to proper cell growth and migration, nutrient flow, vascularization and spatial organization. It should be about 90% to better accommodate osteoblasts or osteoprogenitor cells, enhance cell proliferation and differentiation and increase bone tissue formation. Nevertheless, porosity degree shouldn't be excessively high, otherwise mechanical stability of the template could be adversely affected.

Correlated to the previouses, there are the *interconnectivity* and the *open pore structure*. Scaffolds with an open pore, fully interconnected geometry grant the neovascularization in and around the defective tissue within few weeks after implantation and therefore nutrients but also metabolic waste can be transported more effectively into and out of the cell-scaffold construct, respectively (Bouler et al. 2017).

Pore size is a very cell-type dependent specification. Acceptable diameters, for bone reconstruction purpose, lie in the range between 10 and 500 μm ; pore size above the range reduces mechanical properties, such as compressive strength, and make the manufacturing technique more complex in order to obtain a reproducible scaffold (Suntornnond et al. 2015). On the other hand, dimensions situated below the range drive cells to occlude the pores, impeding cellular penetration into the inner areas of the structure, extracellular matrix production and neovascularization. Small pores also promote hypoxic conditions and induce first osteochondral formation than osteogenesis. It has been shown that scaffolds with a minimum pore size of 150 μm and a mean pore size of 300 μm are optimal for bone tissue formation. It is believed that a structure with a mean pore size greater than 300 μm promotes angiogenesis thereby improving bone regeneration (De Witte et al. 2018).

Some studies (F. Carfi Pavia et al. 2008; Bouler et al. 2017) recommended that the fabrication of multi-scale porous scaffolds, involving both *microporosity* and *macroporosity*, can perform better than a scaffold having only a macro-porous structure. In fact (considering the dimension range initially described), while larger pores must be included in the design to augment new bone formation, to faster bone ingrowth and driving the creation of capillaries, smaller pores (about 10 μm) allow an immediate protein and cell adhesion, migration, osteointegration and the resultant bone tissue ingrowth and vascularization (Amini, Laurencin, and Nukavarapu 2012). Pore shape may play as well a profound role on cell attachment and long-term survival on the scaffold's surface.

Chemical *surface properties* modify cellular adhesion and proliferation. From the chemical point of view, scaffold's material is able to get better or worse cell seeding into itself; even the variations in surface charge may influence cell spreading, surface affinity and changes in phenotypic expression. The **osteo-conduction** is surely the best properties for a bone scaffold. An osteoconductive material will lead to significant bone formation in vivo environment (Kneser et al. 2002). Furthermore, surface irregularity and roughness favour cell migration during the tissue regeneration and promote deposition of a mineralized matrix by osteoblast-like cells, too (Font Tellado, Balmayor, and Van Griensven 2015).

Mechanical properties of a scaffold should be as close as those found in the host tissue; however, the wide variability in mechanical properties, often linked to tissue geometry, make it difficult to elect an ideal scaffold. In For instance, scaffolds are designed to repair the human cancellous bone should have a compressive strength between 2 and 12 MPa and an elastic modulus between 0.1 and 5 GPa (De Witte et al. 2018). Instead, among all ligaments of knee, anterior cruciate ligament (ACL) presents the highest mechanical properties. Therefore, scaffold for anterior cruciate ligament (ACL) regeneration should present value of elastic modulus (65-447 MPa), ultimate tensile strength (13-46 MPa) and ultimate strain (15-44 %).

Finally, as reported before, a main trait of scaffold is its *sterilizability*. The sterilization procedure is easy but its effect must be accounted for in the scaffold design process. Indeed, treatments like autoclave, UV, gamma or ethylene oxide are often poorly

adapted to polymeric scaffolds due to their low melting points and degradation mechanisms. Furthermore, some methods can provoke undesirable polymer chain scission or crosslinking with an alteration of the final properties.

1.5 Suitable materials for BTE scaffolds

The selection of the material for bone tissue engineering scaffolds is critically important in determining almost all the properties listed above: indeed, material's features are strictly related with scaffold's characteristics. Accordingly, a review of the materials proposed for the repair and regeneration of damaged or traumatized bone tissues is mandatory. Several materials are used for biomedical application such as natural and synthetic polymer, metals and inorganic materials (ceramics, glasses).

Despite metals and ceramics have mechanical properties higher than or similar to those of bone tissue, they are non-resorbable and have limited processability. However, some studies reported good results in terms of osteoblasts cell attachment and proliferation when seeded into porous metallic scaffolds, predominantly made in titanium and tantalum, as well as high compressive strength and excellent fatigue resistance. But, unlike bioresorbable polymeric materials, they are permanent, present certain challenges related to metal ion release and biomolecules cannot be integrated into them (Xue et al. 2007). Ceramics materials (hydroxyapatite or bioactive glass) are also combined with polymer to produce composite scaffold able to mimic mechanical and biological response of native tissue (Ramesh, Moratti, and Dias 2017). Mechanical properties of

human bone vary according to location and function. Again, the restorative scaffold's mechanical properties should be modulated or tailored to match the demands of the defect site, to decrease or avoid complications. The scaffold's material composition largely influences its mechanical properties.

In Figure 1.12 the elastic modulus and the compressive strength of dense bioactive ceramic, biodegradable polymers, cancellous and cortical bone are compared with porous monophasic scaffolds and composites. Some dense polymers match cancellous bone properties and approach cortical bone properties. Dense ceramics (HA, calcium triphosphate) possess elastic moduli and compressive strength similar to human cortical bone; however, they are brittle and display slow degradation rates (Amini, Laurencin, and Nukavarapu 2012). Porous scaffolds however are at least one order of magnitude weaker than cancellous bone and orders of magnitude weaker than cortical bone.

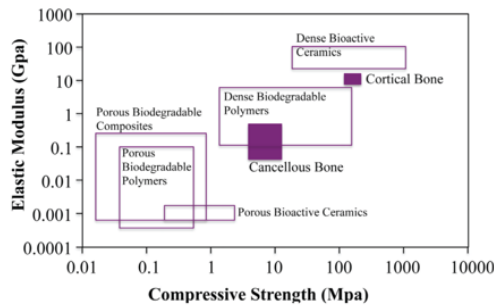


Figure 1.12 Elastic modulus versus compressive strength values of various BTE biomaterial classes compared to human bone, from Amini et al., 2012

Specifically, in this paragraph a short description of natural and synthetic polymers is reported. Instead, next chapter will mainly deal with the materials (polymer and ceramics) used in the experimental phase of this work.

1.5.1 Natural polymers

Natural polymers, such as fibrin, hyaluronic acid, chitosan and collagen exhibit good biocompatibility, osteoconductivity and low immunogenicity. Their possess of innate biological cues that favours cell attachment and their promoting of a chemical stimulus response when being implanted in vivo lead to very good results in terms of biocompatibility and tissue-genesis support (Roseti et al. 2017). However, there are common disadvantages such as the difficulty of processing them into desired shapes or architectures, the non-easily controlled rate of degradation and low mechanical stability.

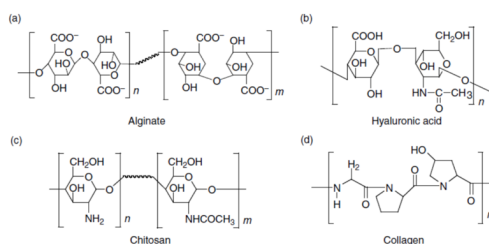


Figure 1.13 Chemical structure of some natural polymers

A several studies (Suntornnond et al. 2015; Asghari et al. 2017) reported as common materials used for bone tissue engineering the follow:

Alginate: it is a polysaccharide extracted from marine algae, completely soluble in water. Alginate is a polyanionic saccharide composed by a mannuronate block and a gulunorate one, whose lengths and proportions establish mechanical properties of the natural polymer. To form a cross-linked hydrogel the exposition to a divalent cation (usually Ca^{2+}) is required. Alginate-based scaffolds have been shown supporting chondrogenesis (i.e. differentiation of stem cells into chondrogenic cell lineage) and promoting the formation of hyaline cartilage (C. C. Wang et al. 2011).

Hyaluronic acid: it is a water-soluble polysaccharide composed by a glucuronate sugar unit and a glucosamine one, abundant within the cartilage ECM. Hyaluronic acid (also called hyaluronan) needs crosslinking to be useful as a scaffold matrix material and its nonimmunogenic and no adhesive properties make it an attractive option for biomedical application (i.e. dermal filler, osteoarthritis treatment, scaffold for chondrocytes growth)(Suntornnond et al. 2015).

Collagen: Collagen-based materials are considered to be favourable for both cartilage and bone scaffolds since collagen is the major component in ECM (collagen type II in articular cartilage and collagen type I in bone). Collagen is composed of three polypeptide chains that intertwine with one to another to form a triple helix. It is characterized by high mechanical strength, good biocompatibility, low antigenicity and

ability of being crosslinked, although some disadvantages of collagen include difficulty in processing, in sterilization and also in controlling the rate of degradability (Sampath et al. 2016). Collagen plays a key role in promoting the osteogenic differentiation of bone progenitor cells, thereby promoting cell growth and mineral production (Dong and Lv 2016). Collagen scaffolds can be prepared either by decellularization of a collagen matrix, retaining the tissue shape and ECM structure, or by extraction, purification and polymerization. However, the mechanical properties of collagen-based scaffolds are generally not sufficient to withstand load-bearing in joints (Camarero-Espinosa et al. 2016).

Chitosan: it is a linear polysaccharide that is derived from natural chitin, which is composed of glucosamine and N-acetylglucosamine. Chitosan molecule has amino and hydroxyl groups which can be modified chemically providing a high chemical versatility and is metabolized by certain human enzymes. Chitosan is widely studied as injectable hydrogel for cartilage repair (Tan et al. 2009) due to its structure similar to glycosaminoglycans that can be found in ECM of articular cartilage, which influence the modulation of morphology, differentiation and function of chondrocytes. A lot of application as scaffold for bone regeneration are reported in literature (Suntornnond et al. 2015).

1.5.2 Synthetic polymers

The use of synthetic polymers is justified not only by their properties (porosity, degradation time, and mechanical characteristics), but also by the fact that it is possible to design the polymer specifically tailored to the application. The most commonly used are poly-(α - hydroxyacids), such as poly-glycolic acid (PGA) and poly-lactic acid (PLA), and poly-(ϵ -caprolactone). The degradation products of these polymers are glycolic acid and lactic acid, which are naturally found in the human body and therefore are removed by natural metabolic path-ways (García-Gareta, Coathup, and Blunn 2015). These materials are approved by the U.S. Food and Drug Administration (FDA) for human clinical applications (Iqbal et al. 2019). The main disadvantage of synthetic polymers is their poor mechanical properties, also a high local concentrations of acidic degradation products can affect cell differentiation *in vitro* and could induce an inflammatory response *in vivo*. Moreover, dissolution of the polymer is often accompanied by breakup into smaller particles which then dissolve inducing an inflammatory reaction (Sa and Kim 2013). Below, main polymeric materials employed for bone tissue engineering scaffold are presented:

Poly(α -hydroxy esters): They are biocompatibility, biodegradability, and easy processing ability. Polylactide (PLA), polyglycolide (PGA), and their copolymer poly(lactide-co-glycolide) (PLGA) belong to this category (Figure 1.14). PGA, PLLA and PLGA are used in the bone and ligament regeneration due to their capacity to sustain cells attachment and proliferation. Furthermore, the tensile strength highest is for PGA (7

GPa) while for PLLA and PLGA is 2.7, 2.0 GPa respectively (Petrigiano, McAllister, and Wu 2006).

PGA is a linear aliphatic polyester is formed by ring-opening polymerization of cyclic diesters of glycolide. Due to the crystallinity of the glycolide, PGA has a high melting point (185 to 225°C) and low solubility in organic solvents. PGA degradation leads to the formation of glycolic acid, and at high concentrations, the acid could lower the pH of the surrounding tissue causing inflammation and possible tissue damages (Hassan, Sun, and Sultana 2014). Poly(glycolic) acid polymers have been investigated as scaffolds in order to support various types of cell growth such as bovine chondrocytes (T. H. Kim et al. 2014), myofibroblasts and endothelial cells (Marrella et al. 2016).

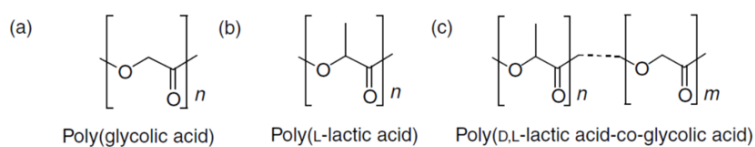


Figure 1.14 Chemical structure of some synthetic polymers

PLLA is structurally similar to PGA, with the addition of a pendant methyl group. PLLA has a lower melting temperature (170 to 180 °C) and an increased hydrophobicity (Morent et al. 2011) that allows protein adsorption and cell adhesion making it a suitable material for scaffolds (Rezwan et al. 2006). However, PLLA has been found to support

various cell types such as: nerve cells (Pav, Carfi et al. 2019) and a lot of application with chondrocytes seeding (Ghosh et al. 2008).

PLGA, poly(D,L-lactic acid-co-glycolic acid), is a product of copolymerization of poly(glycolic) acid and poly(lattice) acid. This copolymer is amorphous, and it has a lower degradation rate and mechanical strength with respect to the single PLLA. It is possible to control the degradation rate by adjusting the ratio of PLLA/PGA during its preparation. PLGA has been extensively researched as a tissue engineering scaffold material and has shown a good compatibility with many cell types. Many studies have been conducted with chondrocytes and osteoblasts resulting in a good expression of collagen, fibronectin, laminin, and a variety of other extracellular matrix proteins, indicating a proper cell growth (Rezwan et al. 2006).

Poly(ϵ -caprolactone)(PCL): it is a biocompatible, bioadsorbable, and biodegradable polyester. PCL is synthesized by ring-opening polymerization of caprolactone monomer (Morent et al. 2011). PCL is a hydrophobic semi-crystalline polymer with low melting point (60°C), low glass transition temperature (-60°C) and Young's modulus of 0.4 GPa, but has good solubility in most of organic solvents. The degradation of PCL occurs by bulk or surface hydrolysis of these ester linkages, resulting in a by-product of caproic acid. PCL degrades at a slow rate with results showing that it can persist in vivo for up to 2 years, but it is possible to increase the degradation rate copolymerizing PCL with collagen, polyethylene-oxide and other polymers (Dulnik et al. 2016). Furthermore, it is easily processable and it has ability to form a blend with other polymers such as PLLA,

PLGA (Rezayan et al. 2017). Thanks to this property PCL has become an attractive component for polymeric scaffolds (Tillman et al. 2009; Ratheesh et al. 2017), whereby it has been used in many forms as TE scaffolds in the form of film, membrane, or even hydrogel (Suntornnond et al. 2015; Petrie Aronin et al. 2008).

Polyphosphazenes: they are a class of polymers formed from an inorganic backbone containing nitrogen and phosphorous atoms. Polyphosphazene is hydrophobic and typically undergoes hydrolytic surface degradation into phosphate and ammonium salt by-products. Taking into account that it is possible to synthesize a lot of polyphosphazenes, due to the numerous types of hydrolytically labile substituents that can be added to the phosphorous atoms, the properties of the resulting material are highly dependent on the side substituents and their ratios. Nevertheless, thanks to the nature of the polyphosphazenes, many of them display a slow degradation rate in vivo. This polymer has been particularly investigated as a biomaterial for tissue engineering due to its high strength and surface degradation properties. Laurencin et al. showed that osteoblasts were successfully seeded and proliferated on a three-dimensional polyphosphazene scaffold (Andrianov and Langer 2009).

1.6 Scaffold fabrication technique

After selecting the proper scaffold material, an adequate processing technique has to be chosen: it should ensure the production of a scaffold having the desired characteristics for BTE. In the field of bone scaffold fabrication technologies, two main macro-groups can be identified: *conventional* fabrication techniques and *solid-free form (SFF)* fabrication techniques. The methodologies belonging to the first class make it possible to create a bulk or porous scaffold's structure, interconnected or not, without any long-range channelling microstructure with limited ability to control shapes. In addition, the use of organic solvents may compromise cell viability or functions, even when only residues remain. In contrast, SFF techniques are based on computer-generated models which allow to obtain a template presenting a highly precise geometry. A more punctual control of porosity, pore size and mechanical properties permits to better mimic natural bone tissue structure. Moreover, toxic organic solvents are not used, allowing a significant enhancement in scaffold biocompatibility (Roseti et al. 2017). The most famous conventional methods are summarized in Table 1.1 and for each one advantages and disadvantages are highlighted. However, a short description of these processes will be made below.

Table 1.1 Advantages and Disadvantages of Conventional Processes for Fabricating Scaffolds

TECHNIQUES	ADVANTAGES	DISADVANTAGES	REF
<i>Solvent casting and particulate leaching</i>	produce highly porous scaffolds (93%)	limited mechanical property; residual solvents and porogen material	Sola et al. 2019
<i>Gas foaming</i>	organic solvent free process, porosity, and pore size can be controlled in scaffolds	yields structure with large unconnected pore and nonporous external surface; limited mechanical property	Dehghani and Annabi 2011; Ji et al. 2012
<i>Phase separation</i>	process conducted at low temperature; incorporation of bioactive molecules	solvent selection and processing temperature are crucial	Mannella et al. 2014
<i>Melt molding</i>	Independent control over porosity and pore size	possibility of residual porogen and high processing temperature	Correlo et al. 2009
<i>Freeze-drying</i>	Hight temperature and separate leaching process are not required	long processing temperature and small pore size	Brougham et al. 2017

Solvent casting/particulate leaching

It is the best-known and most used technique, probably because of its simplicity. It consists in the dispersion of some soluble salts, used as porogens, in a polymeric solution and in the subsequent evaporation of the solvent and salt leaching, in order to obtain a porous structure (Figure 1.15 A). This technique was applied to fabricate biodegradable scaffolds mimicking trabecular bone. The simple operation, good control of pore size and porosity through salt/polymer ratio and size of salt crystals make solvent casting/particulate leaching one of the most used processes for bone scaffold fabrication. Nevertheless, it also has certain problems related to the cubic crystals of the salts that limit the pore shape and, more importantly, to the difficulty in removing salt particles from the inner areas of the construct and to the insufficient interconnectivity. This method is widely used to produce membrane with thickness in range 35-180 μm . Disadvantages of this technique are represented by the fact that the process can only form simple shape scaffolds (flat sheets and tubes) and that the residual solvent could be harmful to cells (Subia, Kundu, and Kundu 2010).

Gas-foaming

In the gas-foaming process (Figure 1.15 B), the porosity-inducing agent are carbon dioxide or nitrogen, avoiding the use of an organic solvent, often toxic. The compressed gas is forced to enter inside the solid polymer foam, favouring CO_2 saturation of the foam. Thermodynamic non-equilibrium is then imposed, sudden decrease in the solubility of gas in the polymer brings the pressure of CO_2 back to atmospheric level resulting in nucleation and the growth of gas bubbles inside the polymer matrix, hence forming the

scaffold's void fraction. The pore size into range of 100–500 μm (Ratheesh et al. 2017) and a porosity up 85% (Roseti et al. 2017) achieved with this technique would enable scaffolds to be used in the bone regeneration applications, but often the surface of the construct is pore-lacking and the pore structure is closed (only 10-30% of pore interconnection).

Emulsion freeze-drying

The method (Figure 1.15 C) is carried out by preparing an emulsion between a polymer solution and water; thereafter the mixture is rapidly cooled to solidify the structure and ice is induced to sublimation through a drying step performed in a vacuum desiccator. At the end scaffolds with porosity higher than 90% and a wide range of pore size can be produced, but, as in the previous technique, the final structure has a closed pore system (Sampath et al. 2016).

Electrospinning

Electrospinning (Figure 1.15 D) is based on the deposition of polymer fibres in a target substrate assisted by an electric field imposed between the polymer solution contained in a syringe and the fibre collector. The polymer solution is injected with an electrical potential in order to alter the charge of the material; this imbalance is able to overcome the surface tension present at the tip of the syringe and, consequently, an electrically charged polymer jet flows outside the syringe. During its electric field-oriented pathway, the fibre loses the solvent amount by evaporation, thus leaving a pure polymer fibre deposited on the collector. This process is very versatile in terms of fibre diameter: the thickness of the fibres can be tuned from several microns to hundreds of

nanometres, giving in this manner a very effective pore interconnection. However, only sheet and cylinder-shaped scaffolds were obtained since now.

Phase separation technique

This is the most versatile methods among of techniques to fabricate polymeric scaffolds. Thermally induced phase separation (TIPS) has distinct advantages such as a pore size distribution and high reproducibility (Molladavoodi et al. 2013; Kim et al. 2017; Carfi Pavia et al. 2019). The phase inversion method can proceed in several different ways. In the TIPS process, the temperature decrease ensures thermodynamic instability of the polymer solution, while in DIPS, this phenomenon is induced by adding a certain amount of non-solvent species which makes immiscible the solution at constant temperature. The TIPS consists in leading a temperature-guided phase separation on a ternary solution usually composed by a polymer, a solvent and a non-solvent of the polymer. The temperature decrease provokes the system to enter inside the miscibility gap and to separate into two phases, each one containing a different concentration of polymer. The porogens, in this case, are droplets of polymer-lean phase that nucleate and grow within the continuous polymer-rich phase; after a freezing step, the droplets containing the polymer-lean phase can be removed through a washing and drying process to achieve the final porous structure. The process is very simple and its flexibility allows to design scaffolds with distinct properties by changing some process parameters like temperature decrease, polymer concentration and phase separation time. Scaffolds prepared with this procedure possess a proper mechanical stability and composite scaffolds also can be prepared via TIPS. The only drawback of this methodology is that it

gives scaffolds with pore size ranging from 10 to 100 μm ; these dimensions, although at the lower limit, were demonstrated to be acceptable for bone cell implantation.

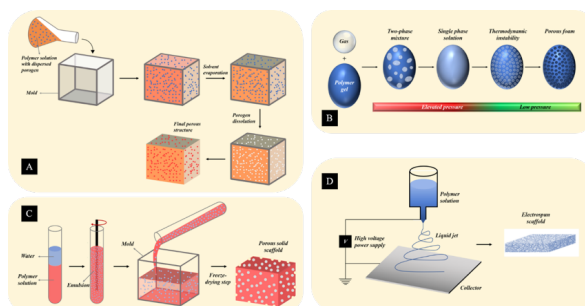


Figure 1.15 Some of the conventional scaffold fabrication techniques; (A) Solvent casting/particulate leaching; (B) Gas foaming; (C) Emulsion freeze-drying; (D) Electrospinning, adapted from Sampath et al, 2016

On the other hand, some SFF techniques are 3D printing and Fused deposition modelling (FDM).

3D printing

The first SFF technique proposed for biomedical and tissue engineering purposes was 3D printing: it involves inkjet technologies to print a template made of several overlapped powder material layers. By printing layer after layer (Figure 1.16 A), each one bound to the other one through a liquid jet, it is possible to reproduce the full scaffold's structure previously designed in a CAD model. Different morphologies were tested with 3D printing, as well as different materials, showing good interactions with

cells. At the same time, some limitations make this technique less attractive for BTE, such as the jet size, which is not still ready to produce a fine microstructure; additionally, mechanical properties of 3D printed scaffolds have to be improved (Chia and Wu 2015).

Fused deposition modelling (FDM)

Based on the same concept of layer by layer deposition, fused deposition modelling (FDM) exploits the thermo-plasticity of a polymer monofilament to build the monolayer (Figure 1.16 B). The filament is guided by two rollers to enter in the head of the machine, where it is semi-molten and extruded from a nozzle tip. While the FDM head moves along x and y axes, the platform in which the filament is deposited moves in the z direction. One of the advantages with respect to the conventional methods is to better control both the internal and the external morphology of the scaffold with extreme accuracy; on the other hand, it is difficult to incorporate eventual growth factors within the filament or jet because the high processing temperatures would destroy them (Ratheesh et al. 2017).

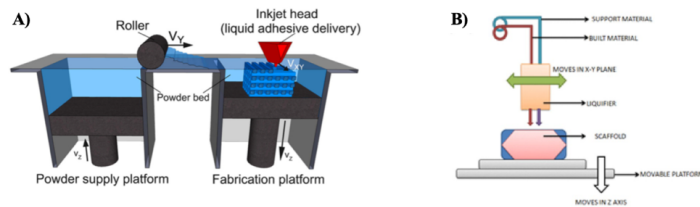


Figure 1.16 A) 3D printing; B) Fused deposition model for the fabrication of scaffolds

1.7 Cell line for BTE and cells-scaffold interactions

The choice, isolation and expansion into high numbers of a reliable cell type is indispensable for BTE field. Ideally, a cell source should be appropriate for expansion to higher passages, non-immunogenic and similar to the tissue to be regenerated in terms of protein expression pattern.

One of the most obvious choices is that of isolating and expanding *in vitro* osteoblast cells, collected from patients' biopsy samples. Their capacity to vastly regenerate bone tissue defects is, in fact, well known. Overall, the use of osteoblasts in BTE is really diffused because it doesn't provoke any problem of immune rejection. Expanded osteoblast cultures were shown to heal a small bone defect after the implantation, maintaining their repairing ability and inducing osteogenic tissue. In spite the osteoblasts are capable to proliferate in culture without losing their viability, they need a considerable number of cell divisions to rebuild the damaged tissue, so tissue regeneration requires much time (Ishaug et al. 1997). Early investigation focused on fibroblast cell lines with the ideal cell source for ACL regeneration could show robust proliferation and possess the potential to elaborate ECM in an organized these differentiated cells possessed the phenotypic characteristics necessary for organized collagen synthesis and ligament formation. Unfortunately, proliferation of adult fibroblasts depend on the tissue culture substrate and they are relatively quiescent and have limited potential for further differentiation (Petrigliano, McAllister, and Wu 2006).

A very valid and promising route appears to be stem cell biology. Stem cells are undifferentiated cells with various degrees of differentiation potential. In the last years,

stem cells are introduced in BTE thanks to their high proliferation capability, self-renewal, multilineage differentiation and thus regeneration of tissues. The source of osteoprogenitor cells is bone marrow. Indeed, these cells named *Mesenchymal Stem Cells (MSCs)* are commonly extracted through a marrow aspiration procedure. In order to induce osteogenic differentiation, cultured MSCs are treated with a medium including dexamethasone, β -glycerophosphate and ascorbic acid. Dexamethasone activate cell differentiation but, if added at higher concentrations than suggested, has negative effect on cell proliferation. B-Glycerophosphate, instead, brings to the maturation of the phenotypic matrix by enabling mineral formation. After being exposed to these stimulating factors, MSCs go through a development featuring the synthesis of alkaline phosphatase, the expression of bone matrix protein mRNAs and the deposition of calcium crystals. More recently, co-cultures of various cell lines were tested to improve the results for *in vitro* BTE; among these, osteoblasts and osteoclasts, osteoblasts and endothelial cells and the co-culture of differentiated and stem cells attracted major interest. One of the advantages of co-culture is the synergy established between the two cell types: for instance, while stem cells provide a constant cell renewal, the other cell population induces differentiation of the formers via efficient signal transduction pathways. To avoid the fusion of different cell types caused by the intimate physical contact, several alternative co-culture arrangements were designed, like the culture of two cell lines in one chamber, separated by a selectively permeable membrane and cells cultivated in two different chambers exchanging the same medium secreted by cells (Bischel et al. 2016; Kang, Zeng, and Varghese 2018).

Chapter 2

2 Aim of this work and strategies for preparing composite scaffold

In this chapter, the aim of this PhD thesis is described. Starting from literature review, the choose of materials and techniques employed to produce composite scaffold for bone tissue regeneration will be reported. That will be validate in the experimental section verifying that scaffold structural features are comparable with the native tissue to substitute.

2.1 Aim of research work

This research work fits in the bone tissue engineering field. As illustrated in scientific literature, bone disease such as osteoporosis and osteoarthritis, or even removal of a bone tumor are increasing. Unfortunately, in the case of tissues with limited vascularization for healing, surgery is required for the grafting of implants such as prostheses.

The aim of this PhD project is proposed alternatives to standard procedures providing medical support in order to improve outcomes of surgical procedure. The idea is founding a good methodology and material combination to produce biodegradable and

biocompatible polymer scaffolds to overcome problems like rejecting risk due to a high inflammatory response.

As mentioned in literature review, the most common materials used are hydroxyapatite and bioglass, which show positive effects since they induce cell differentiation and also present osteoinductive activity. Unfortunately, ceramic scaffolds have got low toughness, they are brittle and sometimes the degradation rate is too fast.

This study suggests to overcome these limits by combining these materials as fillers in polymer matrices. Among all polymers, L-poly-lactic acid (PLLA) was chosen for its high biocompatibility and good resistance in physiological environment. The main disadvantages of PLLA are degradation time too long and also the high deformation during compressive tests, anyway this could drive to a difference versus the native bone tissue mechanical behavior.

The combination between PLLA-filler could improve the mechanical properties of the polymer alone, taking the best properties of the two. In addition, reinforced porous structures can be obtained with greater bioactivity and controlled reabsorption rates.

The goal is understanding how each ceramic (HA, BG or both) can influence the behavior of the composite scaffold in biological environment, material properties and biological response. All these studies will be carried out separately for each scaffold produced: PLLA-HA, PLLA-BG and PLLA-HA-BG.

As scaffold preparation technique, thermally induced phase separation (TIPS) was used. It is different from the other technique usually proposed in literature, but was

chosen because it allows to obtain porous structures with a high degree of interconnection starting from a polymeric solution with filler. The purpose is to prepare a composite material that mimic as closely as possible the bone tissue in terms of morphology and properties.

Other problems, commonly diffused in the young population are the ligament injuries. Furthermore, looking for a good alternative to traditional methodology proposed for ligaments regeneration was also investigated in the work, given the unavailability of a material with mechanical properties similar to ligaments and the low surgical efficiency as detailed before.

The PLLA was used always as material according to its properties, but in this case also a combining with the BG was investigated. The idea to use BG as filler was born thank to its ability to reduce the inflammatory response. Since is known that the ions release of BG stimulate cell proliferation, from this study this evidence could be confirmed for the anterior cruciate ligament cells. As fabrication technique, the Diffusion Induced Phase Separation (DIPS) because allows to produce tubular porous scaffolds with homogeneous microporous structure needed to allow the complete cell scaffold colonization.

Below, the main characteristics of two fabrication techniques (TIPS, DIPS) and the material chosen to produce the scaffold are treated in details, focusing on the description of the solution thermodynamics and solidification kinetics.

2.2 Thermally Induced Phase Separation (TIPS)

Phase separation methods are based on the same thermodynamic principle of inducing a stable polymeric solution to an instability condition.

At constant pressure and temperature, thermodynamic equilibrium of a system is obtained by the value of the *Gibbs free enthalpy* (G), a state function defined as:

$$G = H - T S$$

where H is the enthalpy of the system, T is the temperature and S is entropy. The free enthalpy variation of a process at constant temperature can be expressed as well:

$$\Delta G = \Delta H - T \Delta S$$

The values of enthalpy and entropy variation establish whether a process, such as a chemical reaction or a mixing process, is spontaneous or not. In particular, for a mixing process, the equation is represented with the relative entities of mixing:

$$\Delta G_m = \Delta H_m - T \Delta S_m$$

The mixing of two components, for example polymer/solvent, will be spontaneous only if the free enthalpy of mixing is negative ($\Delta G_m < 0$). Usually, for polymer solutions, the entropic contribution is negligible (as it will be discussed later) and thus the miscibility of the two species will only depend on the enthalpy of mixing.

In a two-components system containing n_i moles of species i and n_j moles of species j , the infinitesimal variation of the free enthalpy of mixing, at constant pressure and temperature, is given by:

$$dG = \left(\frac{dG}{dn_1} \right)_{T,P,n_2} + \left(\frac{dG}{dn_2} \right)_{T,P,n_1}$$

Chemical potential of a generic component is defined as the partial molar free enthalpy of the system; with this notation, the above equation can be written in a different way:

$$\mu_i = \left(\frac{\partial G}{\partial n_i} \right)_{T,P,n_j} \rightarrow dG = \mu_1 dn_1 + \mu_2 dn_2$$

For a pure component, the chemical potential may be written as μ_i^0 . The free enthalpy G_m of a mixture consisting of two components is obtained with the sum of the chemical potential of each component. If G_m is expressed per mole of mixture, as a specific entity, then the following relation can be derived:

$$G_m = x_1\mu_1 + x_2\mu_2$$

with x_1 and x_2 representing the mole fractions of species 1 and species 2, respectively. In Figure 2.1, the chemical potential values of the two species in a mixture can be observed, as well as the chemical potentials of the two pure components.

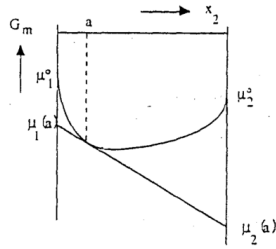


Figure 2.1 Solution's free enthalpy diagram as a function of the composition at temperature T .

When two species are mixed, the variation of free enthalpy of mixing associated with their interaction is (at constant pressure and temperature):

$$\Delta G_m = n_1 \mu_1 + n_2 \mu_2 - n_1 \mu_1^0 - n_2 \mu_2^0$$

By introducing the chemical potential difference of each species as the difference between the chemical potential of the species in the mixture and the chemical potential of the same species considered as a pure component, a shorter form of this equation can be written:

$$\Delta G_m = n_1 \Delta \mu_1 + n_2 \Delta \mu_2$$

Because the chemical potential of a species in the mixture is known if the chemical potential of pure component, molar fraction and temperature are known, variation of free enthalpy of mixing per mole of solution can be derived in this way:

$$\mu_i = \mu_i^0 + RT \ln x_i \rightarrow \Delta G_m = RT(x_1 \ln x_1 + x_2 \ln x_2)$$

In this last equation, an ideal solution is assumed: this hypothesis leads to approximate the activities of the species, a_i , with molar fractions, x_i . Since a logarithm of a fraction always gives a negative number, it can be deduced that, for any ideal solutions, ΔG_m is negative and hence the components always mix spontaneously. Furthermore, for ideal solutions, enthalpy variation is zero and free enthalpy variation is only function of ΔS_m :

$$\Delta S_m = - \frac{\Delta G_m}{T} = -R (x_1 \ln x_1 + x_2 \ln x_2)$$

Flory and Huggins studied the solubility behaviour of polymer solutions: these particular solutions differ from low molecular weight solutions because a polymer, being composed of a chain of molecules, occupies a larger space. With the aid of the lattice theory, authors demonstrated that the combinations obtained by arranging all the molecules in a lattice are very low if compared to a low molecular weight solution.

In addition, for polymer solutions, molar fractions don't coincide with volume fractions; volume fractions of a solution containing a solvent (component 1) and a polymer (component 2) are expressed as:

$$\phi_1 = \frac{n_1}{n_1 + N n_2} \quad \phi_2 = \frac{N n_2}{n_1 + N n_2}$$

The terms N takes into account the fact that the polymer is a series of N repeating units. From these relations, considering n_t , equal to $n_1 + N n_2$, as the total number of the

lattice sites, mole numbers of the two components can be yielded as functions of volume fractions:

$$n_1 = \phi_1 n_t \quad n_2 = \left(\frac{\phi_2}{N}\right) n_t$$

If the entropy variation of the solution (no more per mole of solution), expressed in volume fractions, is given by:

$$\Delta S_m = -R (n_1 \ln \phi_1 + n_2 \ln \phi_2)$$

and if ΔH_m can be considered negligible:

$$\frac{\Delta G_m}{RT} = -\frac{\Delta S_m}{R}$$

then, by combining these two equations:

$$\frac{\Delta G_m}{n_t RT} = \phi_1 \ln \phi_1 + \left(\frac{\phi_2}{N}\right) \ln \phi_2$$

For a polymer-polymer blend, N_1 and N_2 coefficients have to be considered; whereas, for an ideal low molecular weight solution, $N_1=N_2=1$. The effect of the chain length of the polymer is well explained in Figure 2.2.

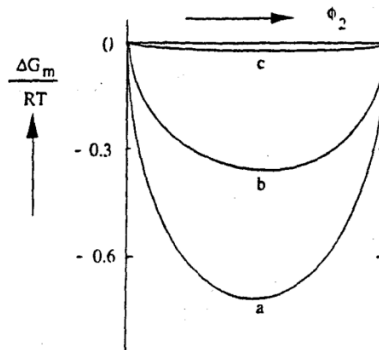


Figure 2.2 Free enthalpy/volume fraction curves for different combinations of N_1 and N_2 : (a) $N_1/N_2 = 1/1$; (b) $N_1/N_2 = 1/1000$; (c) $N_1/N_2 = 100/1000$

It can be seen that the ΔG_m decrease reduces going from two low molecular components to two polymeric components; also, in all three cases, the components are miscible in all proportions. Substances demix only when the enthalpy variation is positive and not negligible, i.e. a positive interaction term exists. Generally, polymers are not miscible each other because the low entropy variation can be balanced and can be overcome by a small enthalpy variation. This is true for solvent-polymer solutions as well. In order to consider non athermal solution, for which $\Delta H_m \neq 0$, a third contribution is added in the definition of free enthalpy variation for solutions, containing the Flory-Huggins interaction parameter:

$$\Delta G_m = RT (n_1 \ln \phi_1 + n_2 \ln \phi_2 + n_1 \phi_2 \chi)$$

It can be assumed that χ contains all non-ideality of a polymeric solution.

By lowering the system temperature, often, the free enthalpy of mixing increases; this trend is shown in Figure 2.3: at temperature T_1 , the system is miscible whatever is the composition, because the tangent to the ΔG_m can be drawn at any composition and it always lies below the miscibility curve. At a minor temperature, T_2 , a different shape of the ΔG_m curve is present, between ϕ^I and ϕ^{II} compositions. It is possible to identify a common tangent to the points of the curve at these compositions: this means that those points are in equilibrium each other, because the chemical potentials are the same ($\partial\Delta G_m/\partial n_i = \Delta\mu_i$).

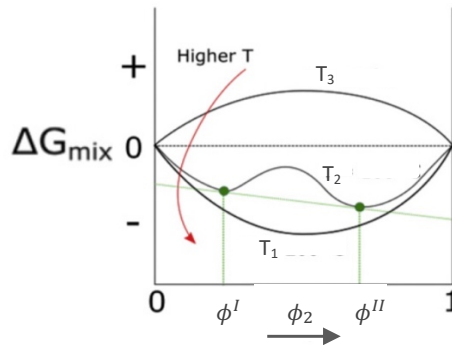


Figure 2.3 Free energy of mixing as a function of composition for a binary mixture at different temperatures: here a thermodynamic instability can be noted between compositions ϕ^I and ϕ^{II} (adopted by Kim et al., 2017)

In general, if the temperature of the system increases, the miscibility of the solution is higher and the two points characterized by the same tangent get closer, i.e. the curve changes curvature for a narrower range of compositions. At a certain high enough temperature, they even tend to coincide in a single point, called critical point. In Figure 2.4A two points of inflections can be appreciated as well, at compositions ϕ^1 and ϕ^2 . In a temperature/composition diagram the locus of the minima of the ΔG_m curve and the inflection points can be drawn: in doing so, binodal and spinodal curves are identified, respectively (Figure 2.4 A). The binodal curve includes within the miscibility gap of the polymeric solution.

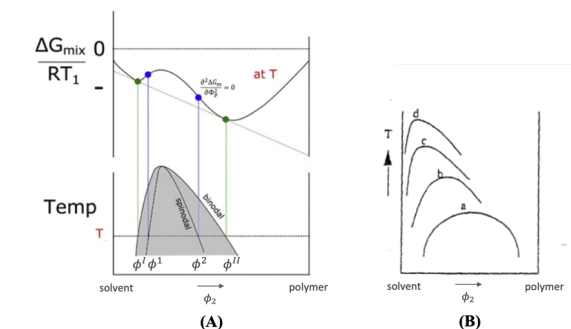


Figure 2.4 A) Correlation between ΔG_m and the temperature/composition phase diagram for a binary polymer-solvent system; B) Trend of the immiscibility region of a binary mixture in function of the molecular weight of the polymer: from curve “a” to curve “d” the molecular weight increases (adopted by Kim et al.,2017).

Normally, the chain length of the polymer determines the position of the miscibility gap of a solution made of a solvent and a polymer: as the molecular weight increases, the curve shifts towards the solvent axis and to higher temperatures (Figure 2.4 B).

A typical polymer-solvent TIPS system can be expressed by phase diagram temperature-composition (Figure 2.5). A semicrystalline polymer is first dissolved in an appropriate solvent. The homogeneous solution can undergo a phase separation by cooling since the affinity between the polymer and the solvent decreases and the polymer precipitates (crystallizes) being below its melting temperature. Cooling the solution, depending on final temperature, two different mechanisms of phase separation can take place: liquid-liquid (L-L) or solid-liquid (S-L) separation that lead to different morphology depending on phase separation pathways. In S-L separation, polymer crystallizes directly from the solution and a powder (sphere-like) morphology is obtained.

In L-L separation, solution phase demixes into a polymer-rich phase and a polymer-lean phase with subsequent solidification and the final structure is results in a dispersion (cellular) or a bicontinuous morphology. The dispersed occurs via a nucleation and growth (NG) mechanism, which take place when the unstable polymer solution spend enough time between the binodal and spinodal curve.

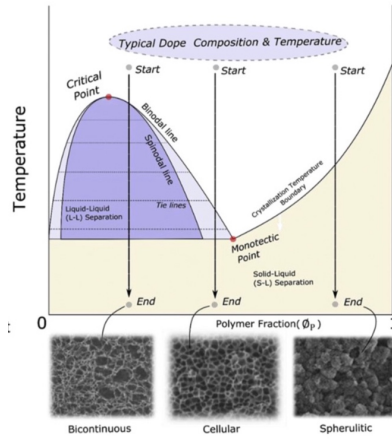


Figure 2.5 Thermally induced phase separation in polymer solutions: S-L phase separation and L-L phase separation with different final morphology, from Kim et al. (2017)

In order to better understand the mechanism of the liquid-liquid demixing process, a thermodynamic stable binary solution at composition A and temperature $T_1 > T_C$ is taken into consideration (Figure 2.6 A). Above T_C , the solution is thermodynamically stable, whereas below T_C , the binodal curve could be crossed and thus provoke system's instability followed by the formation of two different liquid phases. At temperature T_2 , the system lies in an unstable phase because it is inside the miscibility gap: in this zone, liquid-liquid demixing occurs because the free enthalpy of the system at composition between ϕ^I and ϕ^{II} can be reduced if the system demix in a two-phase system of compositions ϕ^I and ϕ^{II} (Figure 2.6 B). Having the same tangent to the ΔG_m curve, the

two phases have the same chemical potential and, consequently, they are in equilibrium among themselves.

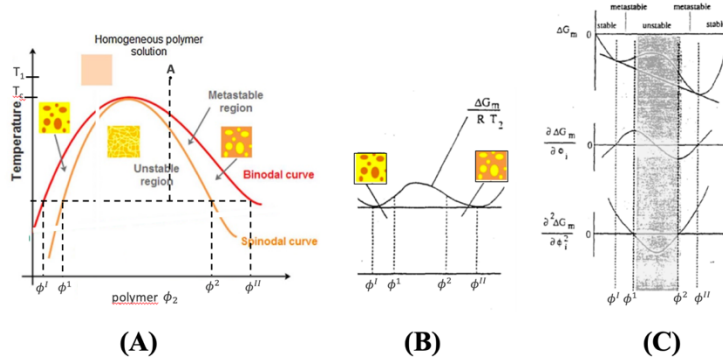


Figure 2.6 (A, B) Demixing process of a binary polymer solution promoted by a decreasing in the temperature; (C) Plots of ΔG_m and its derivatives versus ϕ .

Figure 2.6 C shows some distinct zones in the ΔG_m curve identified from its derivatives: in the whole interval limited by ϕ^1 and ϕ^2 compositions, in which the second derivative of the free enthalpy of mixing is negative, the solution is locally unstable and spontaneously separates into two interconnected phases of composition ϕ^I and ϕ^{II} . The structure obtained through this thermodynamic path is lacy and the process that leads to this final configuration is called spinodal demixing. Within the other intervals between ϕ^I and ϕ^1 and between ϕ^2 and ϕ^{II} , the second derivative of ΔG_m is positive and the solution doesn't demix spontaneously but enters in a so-called metastable state. If, during this path, the system undergoes small fluctuations in composition, it may

suddenly move into an unstable condition and demixing starts. This change of behavior is favored by the generation of stable nuclei; because stable nuclei lower the free enthalpy of the system, in the interval $\phi^I < \phi < \phi^1$, nuclei's composition is close to ϕ^{II} , while in the interval $\phi^2 < \phi < \phi^{II}$ it is close to ϕ^I . Subsequently, nuclei are subjected to a growth process and the composition of the continuous phase tends to that of the other equilibrium phase. In this frame, if the temperature of a polymeric solution with a composition located to the left side of the critical point (diluted solution) is reduced in such a way to bring the solution into the miscibility gap, liquid-liquid demixing will start and nuclei are of composition close to ϕ^{II} . When the thermodynamic equilibrium is reached, a two-phase system is formed involving concentrated polymer droplets with composition ϕ^{II} dispersed in a continuous phase rich in solvent, with composition ϕ^I . In contrast, if the starting solution has a composition located to the right side of the critical point (concentrated solution), the final configuration will be composed of polymer-lean clusters (ϕ^I) dispersed in a polymer-rich continuous phase (ϕ^{II}).

Often, for most polymer solutions, critical point is situated close to the solvent axis, therefore, the binodal curve usually will be crossed in the polymer rich region, resulting in the nucleation of polymer-lean phase. The growth process continues until the continuous phase solidifies; if they grow enough to coalesce each other, when polymer-rich phase solidifies, an open pore structure will be derived.

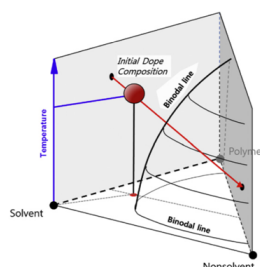


Figure 2.7 Three-dimensional region of immiscibility of the same ternary solution, derived from binodal curves taken at various temperatures, from Kim et al., 2017)

Temperature isn't the only parameter that could be used to cause demixing in polymer solutions: the addition of a third component a solvent less affine to the polymer, called non-solvent, in a polymeric solution, at a certain temperature, can induce phase separation as well. For a ternary system, the immiscibility region is identified as a three-dimensional surface because ΔG_m is a function of both temperature and composition (Figure 2.7). The phase diagram is a ternary diagram and the binodal curve within it is built considering the projection of the points of ΔG_m curve having the same tangent at constant temperature. Even in this case, temperature increase, reduces the miscibility gap is reduced until components are miscible in all proportions. Every isothermal cross-section represents a ternary system with a specific miscibility gap; pure components lie at the corners of the triangle, each binary mixture's composition is defined at the sides, whereas ternary mixtures are points internal to the triangle.

Figure 2.8 shows a ternary system at a given temperature presenting binodal and spinodal curves. The pairs of points which are in equilibrium on the binodal, thus having the same chemical potential, are connected with the tie lines. A system having composition comprised in the two-phase region always lies on a tie line and, because of thermodynamic instability, it splits into two phases, one rich in polymer and the other poor in polymer, with compositions represented by the intersection between the tie line and the binodal.

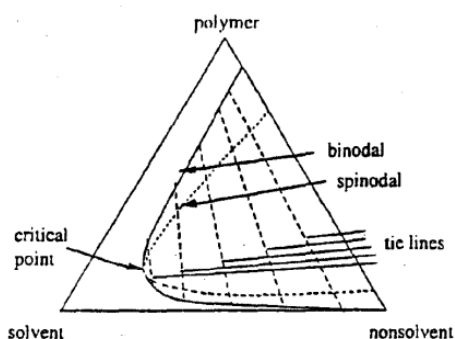


Figure 2.8 Ternary diagram containing a liquid-liquid demixing gap.

In order to induce phase separation on ternary systems, two main pathways can be followed, as described in Figure 2.9. The starting point, in the first case, is a thermodynamically stable (homogeneous) ternary solution. By lowering the temperature, the system may go through the miscibility gap and get into an unstable condition which evolves in the formation of two distinct phases having different compositions (Figure

2.9A). On the other hand, the addition of a non-solvent in a stable binary mixture located in polymer/solvent triangle's side also permits to enter in the two-phase region at constant temperature (Figure 2.9B). As in the case of binary mixtures, even in these cases, it's very important the side, with respect to the critical point, in correspondence of which the miscibility curve is crossed. Since usually the critical point is situated in correspondence to very low polymer concentrations, the miscibility gap often is crossed at compositions above (or in the right side of) the critical point. Consequently, nucleation of polymer-lean phase will occur.

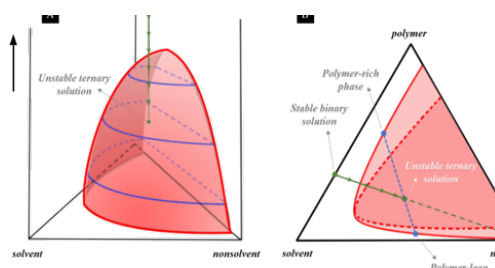


Figure 2.9 (A) Phase separation of a stable ternary solution induced by temperature decrease; (B) Phase separation promoted by the addition of nonsolvent in a stable binary solution.

In summary, the TIPS is a versatile and easy technique that allows to obtain structure with interconnected pores, changing some parameters (Mannella et al. 2014):

- *Polymer concentration*: as the concentration increases, keeping constant the cooling path, pore dimension and porosity decrease accordingly.

- *Polymer molecular weight*: an increase of the molecular weight implies an increase of the viscosity of the solution that plays a crucial role during the formation of the two phases. The foams at higher molecular weight result more homogeneous compared to the others ones.
- *Solvent/non-solvent ratio*: the first attempts to produce scaffold via TIPS were carried out starting from a binary solution polymer/solvent. In this case, the scaffolds present a ladder-like structure due to the solidification of the solvent, and the final scaffold structure is mainly dominated by the progress of the crystallization of the solvent. Inducing a liquid-liquid phase separation by adding a non-solvent allowed a significant improvement in the scaffold architecture together with an increase in the sensitivity of the solution to the tuneable parameters, above all the thermal history
- *Cooling path*: starting from the same solution, the final morphology of the scaffold can exhibit very different features (in terms of pore dimension, distribution, interconnectivity, etc) by simply tuning the cooling path.

2.3 Diffusion Induced Phase Separation (DIPS)

Diffusion Induced Phase Separation (DIPS) was the first technique to be commercially explored, it has been widely studied and applied to produce porous membranes for a large variety of applications. In a typical procedure, the polymer solution is cast on a glass or metal plate and subsequently immersed into a coagulation bath. There are at least three components involved in this process:

polymer, solvent and non-solvent. The phase transition behavior, depending on the interactions of polymer, solvent and non-solvent, is responsible for the pore generation and fixation of the porous structure, which can also be modified by the addition of a second polymer, alcohols, or inorganic salts, into the casting solution. This technique can also be used to realize tubular scaffold in ligaments or vascular tissue engineering thanks to different preparation protocols and suitably modifying the shape of the support (La Carrubba, Pavia, and Brucato 2010).

The main steps of DIPS protocol are: (1) casting of a polymer solution on a support and (2) immersion into a coagulation bath, usually composed by a nonsolvent. The mutual exchange of solvent and nonsolvent will modify the casted solution composition, inducing phase separation when the equilibrium curve is crossed (see Figure 2.10).

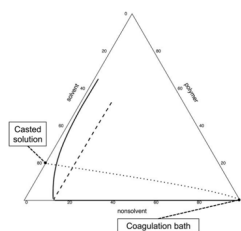


Figure 2.10 Triangular diagram PLLA-dioxane-water

In the triangular diagram (PLLA-dioxane-water), the dashed line is the spinodal line while the black is the binodal curve. The left side of the triangle represents the binary solutions of polymer-dioxane. Putting in contact the binary solution with water, the water diffuses into the binary solution, while dioxane diffuses toward water

as they are fully miscible. The system's concentration changes and so it enters in the miscibility gap. The liquid-liquid phase separation is responsible for the formation of a porous membrane, and it can occur via nucleation and growth and/or spinodal decomposition mechanisms. By varying concentration of polymer solution, coagulation bath concentration, number of bath and time, membrane with different morphology can be produced. As reported by Montesanto et al. 2015, membranes prepared via DIPS show a completely closed and nonporous external surface, owing to a local polymer concentration increase. To overcome this limitation, were investigated how employing a coagulation bath composed of both nonsolvent and solvent at different weight ratio and/or multiple coagulation baths could be change the resulting external surface. The morphologies so obtained are displayed in Figure 2.11.

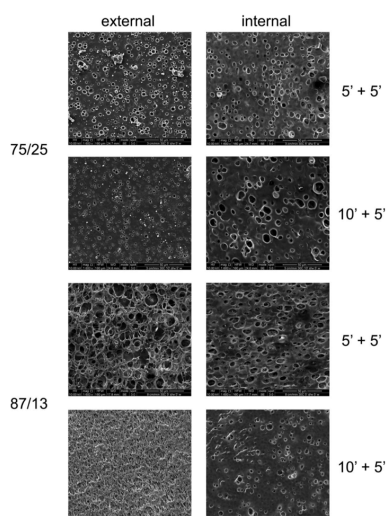


Figure 2.11 Morphology of membrane surfaces obtained with a double immersion, first in a dioxane/water bath, then in pure water, and desiccated at room temperature, from Montesanto et al., 2015

Moreover, during DIPS process is possible modulate the thickness of the final sheet or tube changing the temperature or the pull-out velocity of the support from the solution polymer-solvent. Effects of this two parameters were investigated by Carfi Pavia et al. 2012. Fixing the temperature and the polymer concentration, the thickness increases with the extraction velocity.

2.4 Polymeric solution

In this thesis work, PLLA solutions were prepared to produce biocompatible scaffolds for various applications. Binary or ternary solutions were prepared on the base of the fabrication process followed. A PLLA-dioxane solution with 8% wt/wt polymer concentration was employed in the DIPS process (Carfì Pavia et al. 2012). Instead, a ternary solution of PLLA-dioxane-water (polymer-solvent-unsolvent) was adopted in the TIPS protocol. PLLA concentration in each solution was chosen of 4-6% wt/wt depending on the final scaffold application. The weight ratio 87/13 of dioxane-water was maintained constant for every application. These concentrations were adopted in some works to produce scaffolds for cartilage (Conoscenti, Schneider, et al. 2017) or bone (Ghersì et al. 2016; Carfì Pavia et al. 2018) regeneration.

2.4.1 PLLA

Poly-lactic acid (PLLA) is classified as a linear poly- α -hydroxy acid and it is formed by ring-opening polymerization of L-lactide. Since PLA contains an asymmetric α -carbon, it is typically described by two enantiomeric forms of poly(D-lactic acid) (PDLA) and poly(L-lactic acid) (PLLA). PLA is considered an eco-friendly material since the basic monomer unit (lactic acid) is produced by the fermentation of carbohydrates. The PLA synthesis can take place follow three different paths, illustrated in Figure 2.12 (Sampath et al. 2016).

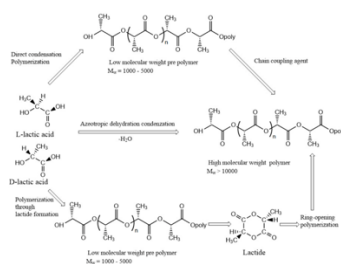


Figure 2.12 Three main routes for obtaining high molecular weight poly(lactic acid) PLA, from Sampath et al., 2016

The lactic acid exists in two configurations: L which is optically active and polarizes light to right (denominated D) and D-configuration polarizes light to the left (Agrawal and Bhalla 2003). D,L-Lactic acid may be converted to suitable high molecular weight polymers through polymerization of their cyclic diesters, or lactides. Poly(D-lactic acid) (i.e. PDLA) and poly(L-lactic acid) (i.e. PLLA) are polymerized by ring opening polymerization of (D)-lactide and (L)-lactide, respectively (Figure 1.14).

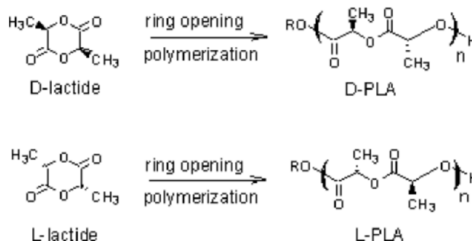


Figure 2.13 Preparation of PDLA and PLLA from respective lactides, from Agrawal et al., 2003

When implanted in the body, PLLA hydrolysis product is lactic acid, a non-toxic substance which is resorbed by the body through the classical metabolic pathways, by transforming it into carbon dioxide and water (Figure 2.14). Together with its intrinsic factors (molecular weight, crystallinity, surface properties), when seeded (*in vitro*) or implanted (*in vivo*), PLLA's degradation behaviour even depends on environmental conditions. In this context, medium exchange, temperature, polymer/host interactions and pH have remarkable effects on degradation kinetics (Ishaug et al. 1997).

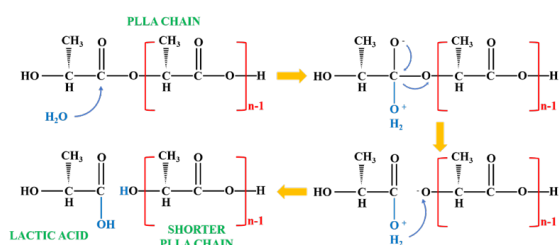


Figure 2.14 Degradation reaction mechanism of PLLA by hydrolytic cleavage

PLLA is a semi-crystalline polymer with a glass transition temperature of 60-65 °C and a melting temperature in range 170-180 °C. This polymer has a slow degradation rate and no significant changes in mass occur for a very long time, total resorption of high molecular weight PLLA can take several years. Besides the slow degradation, PLLA also has a good tensile strength, low extension and a high tensile modulus (Morent et al. 2011). Due to these excellent properties, PLLA can be considered for load bearing applications, such as orthopaedic devices.

High strength PLLA or copolymers as PLGA are also be employed as surgical sutures while other forms of PLLA (composites, porous membranes, sponges) can be used as scaffolding devices for tissue engineering (Sanyanga et al. 2016; Molladavoodi et al. 2012).

It is possible to find in literature different works using blends PLLA with others synthetic polymer such as PCL (Rezayan et al. 2017), PEG. It was demonstrated that in PLLA-PEG scaffold water uptake and degradation rate increase compared with neat PLLA (C. Ji et al. 2012).

In this work, the polymer Poly-L-lactic acid (PLLA, ResomerTN L 209 S) with average number molecular weight of 114000 g mol⁻¹ was used for the fabrication of all scaffolds exanimated.

2.4.2 Dioxane

1,4-Dioxane is the solvent used for scaffold fabrication due to higher affinity with PLLA. Dioxane is a heterocyclic organic compound, a ring of four carbon atoms and two of oxygen. It is produced by the acid-catalysed dehydration of diethylene glycol, derived the hydrolysis of ethylene oxide. Different isomers exist and they are reported in Figure 2.15.

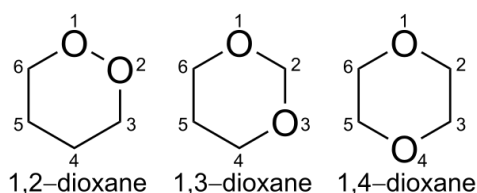


Figure 2.15 Chemical structure of dioxane and its isomers

This organic solvent is totally miscible with water in all composition forming a not-ideal mixture ((E. R. Smith and Wojceichowski 1937; Takamuku et al. 2001)). It is a good solvent for PLLA with a melting point of 11.8°C and boiling point of 101°C. 1,4-Dioxane is mainly used as solvent in the process of scaffold fabrication in BTE (Hua, Nam, and Lee 2001; Frydrych et al. 2015; Frydrych and Chen 2013).

2.5 Solution with filler

An obvious option is to select, as materials for bone tissue engineering, some species already present in natural bone matrix: hydroxyapatite (HA) and other calcium phosphate-containing ceramics, like β -tricalcium phosphate (TCP), are most frequently exploited in bone regeneration strategies.

Repair and regeneration of large bone defects could require as potential solution the use of an appropriate biomaterial that is biocompatible, allows bone ingrowth and shows subsequent degradation of the material. In this framework, the biomaterial used for tissue replacement must be as close as possible to the inorganic or organic components of the

tissue, or both, to be substituted. Bone tissue is a living organ composed by organic and inorganic components. The organic substance (approximately 1/4 to 1/3 of total dry bone weight) consists of more than 90% of collagen and only for a small fraction of cells (2%) and non-collagenous proteins (5%). The inorganic bone mineral (approximately 2/3 to 3/4 of total dry bone weight) is composed of specific phases of calcium phosphate (Ca-P), especially carbonate rich hydroxyapatite (HA) (Yoshikawa et al. 2009). In general, Ca-P materials are crystalline ceramics characterized by a high biocompatibility, osteoconductivity, a variable resorbability and good mechanical properties in compression. Due to these interesting features, many investigators have focused on this class of biomaterials as clinical solution in dentistry and orthopaedics; nowadays, ceramics, such as hydroxyapatite (HA), tricalcium phosphate (TCP) and bioactive glasses (such as bioglass) are widely used in bone tissue engineering. When implanted, these materials promote the formation of a bone-like apatite layer on their surface, leading to bone-bonding behavior and assuring enhanced fixation of the scaffold to the host tissue (Nooeaid et al. 2012).

Since there are many limitations to biomaterial scaffolds, composite scaffolds of both ceramic and polymer are being explored as alternatives to overcome the limitations of low mechanical stiffness seen in polymer-based scaffolds and the brittle nature of calcium/bioactive glasses. A composite material would tailor the desired degradation and resorption kinetics of the polymer matrix, improve biocompatibility and hard tissue integration.

2.5.1 Hydroxyapatite

Hydroxyapatite (HA), whose chemical formula is $\text{Ca}_5(\text{PO}_4)_3\text{OH}$ (Figure 2.16A), being the primary component of bone mineralized tissue, is the most widely used compound due to its excellent osteoconductive and osteointegrative properties. The HA is a basic calcium phosphate of the apatite family hardly soluble under physiological conditions that does not show any toxic, inflammatory and immunogenic response. Impurities like substitution of Ca^{2+} by other metal ions cause variation in solubility and crystal size due to the differences in ionic radius. It is thermodynamically stable at physiological pH and able to form strong chemical bonds with the surrounding bone tissue and hence has a wide range of application in bone repair and regeneration.

HA is generally used as coating of material in titanium (Ratheesh et al. 2017). If treated with or without bone marrow cells, HA based scaffolds give significant results regarding bone regeneration, clearly due to their osteo-conductive and osteo-inductive abilities. Thanks to its ionic, hydrophilic composition, hydroxyapatite has substantial binding properties towards proteins and, hence, may act as a carrier for bioactive peptides and bone growth factors (Ohgushi and Caplan 1999). Mechanical properties of HA, such as good toughness and high resistance to compression, are able to provide the mechanical support at the defect area after transplantation. Pure HA may reach compressive strengths much higher than cortical bone (300–900 MPa), whereas tensile strengths similar and higher than cortical bone (40–300 MPa) have been reported. A disadvantage of HA is the lack of ductility, so it does not exhibit substantial elastic or plastic deformation before

fracturing due to its atomic structure. As a consequence, its brittle nature leads to a difficult processability for this reason a combination with polymer materials was investigated.

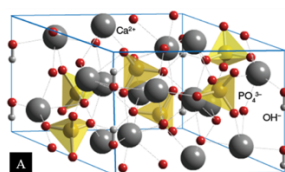


Figure 2.16 Crystallographic structure of hydroxyapatite

Hydroxyapatite particles can be synthesized by several processes: spray drying method (Cholas et al. 2016), wet chemical method (Z. Hong et al. 2004), hydrothermal method, use of sol-gel procedures, solid-state reaction (Sadat-Shojai et al. 2013). By choosing the appropriate fabrication route and experimental condition, hydroxyapatite's structure, including porosity, crystal size, composition, and dissolution properties, can be rendered a very promising artificial matrix for BTE (Eslami et al. 2010).

However, HA has a Ca/P ratio within the range (1.50 - 1.67) in order to promote bone regeneration (Armentano et al. 2010).

There are many techniques actually used to produce composite scaffolds (polymer-HA), such as: electrospinning (Khajavi, Abbasipour, and Bahador 2016) solid freeform fabrication (T. H. Kim et al. 2014), combined centrifugation and freeze-drying (Marrella et al. 2016), gas foaming and salt leaching (Salerno, Iannace, and Netti 2012), thermally

induced phase separation (TIPS) (Vitrano et al. 2018) and solvent casting (Rezwan et al. 2006).

2.5.2 Bioactive glass

The first bioactive glass was invented by Larry Hench at the University of Florida in 1969. He came up with a new biocompatible material using silica (glass) as a base material that could be mixed with other ingredients such as calcium to overcome problems like fibrous encapsulation of bioinert materials (metal, polymer) (J. R. Jones 2013). This material, defined “bioactive”, stimulates a specific biological response at the interface of the material, particularly bonding to host tissue (usually bone). Especially, dissolution products of the glass, i.e. soluble silica and calcium ions, stimulate osteogenic cells and growth factor production with bone matrix produced (Hoppe, Gldal, and Boccaccini 2011; Qazi et al. 2017).

Bioactive glasses exhibit excellent osteoconductivity, bioactivity and controllable biodegradability. These advantages make bioactive glasses promising as scaffold materials for tissue engineering (Chen, Thompson, and Boccaccini 2006). Among the advantages of bioglass over synthetic hydroxyapatite are the biological fixation, and the capability of bonding to both hard and soft tissues, whereas hydroxyapatite binds only to hard tissues. Furthermore, Young’s modulus of bioglass being between 30 and 50 GPa is a great advantage, so nearly that of natural bone. The disadvantage is the low mechanical strength and decreased fracture resistance, limitations that could be overcome by altering the composition or using it in low load-bearing areas (Krishnan and Lakshmi 2013).

Bioglass composition

Bioglass material is composed of minerals that occur naturally in the body (SiO_2 , Ca, Na_2O , H, and P), and the molecular proportions of the calcium and phosphorous oxides are similar to those in the bones (Krishnan and Lakshmi 2013).

The first reported bioactive glass, known as 45S5 Bioglass®, with composition (weight %): 45% silica (SiO_2), 24.5% calcium oxide (CaO), 24.5% sodium oxide (Na_2O), and 6% phosphorous pentoxide (P_2O_5). It has been used as bulk material for the production of medical devices for dental and orthopaedic applications, as particulate in bone-filler defects, as coating on metallic implants and for fabricating tissue engineering scaffolds (Ciraldo et al. 2018).

There are now several types of bioactive glass: the conventional silicates, such as Bioglass 45S5, 13-93; phosphate-based glasses; and borate-based glasses. A silicate bioactive glass, 13-93, has also been receiving interest for bone repair applications because of it has a better ability to be thermally bonded into three-dimensional (3D) architectures without crystallization of the glass (Deliormanlı and Rahaman 2012). Recently, interest has increased in borate glasses (13-93B3), with the same composition as silicate glass but with all the SiO_2 replaced by B_2O_3 . It was recently found that borate glass support the growth and differentiation of human mesenchymal stem cells. Moreover the HA layer formed more rapidly on the borate glass than on 45S5 glass (Ning et al. 2007). Further studies using 45S5 Bioglass® particles have shown encouraging results regarding potential angiogenic effects of Bioglass®, i.e., increased secretion of vascular

endothelial growth factor (VEGF) and VEGF gene expression *in vitro*, as well as enhancement of vascularization *in vivo* (Gerhardt and Boccaccini 2010). Other studies suggest that the osteogenic properties of 45S5 bioactive glass are due to their dissolution products which stimulate osteoprogenitor cells at the genetic level (Radin et al. 2005).

Table 2.1 Composition of 45S5, 13-93 and 13-93B3 glasses (in wt%) used in this research project

Glass	SiO₂	B₂O₃	CaO	Na₂O	K₂O	MgO	P₂O₅
45S5	45	-	24.5	24.5	-	-	6
13-93	53	-	20	6	12	5	4
13-93B3	-	56.6	18.5	5.5	11.1	4.6	3.7

Fabrication techniques for bioactive glasses include both traditional melting methods and sol-gel techniques. The typical feature common to all bioactive glasses, being melt (Schuhladen et al. 2018) or sol-gel derived, is the bioreactivity or bioactivity (Boccaccini et al. 2010). Figure 2.17 shows how varying proportions of the components cause the bioglass to be bioinert, bioresorbable, or bioregenerative. Six distinct areas can be individuated.

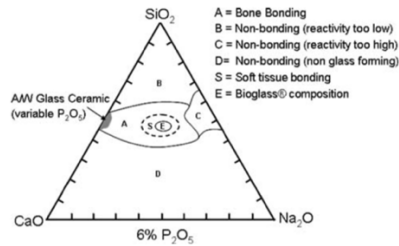


Figure 2.17 Property changes of bioglass materials about bone-bonding, from Hench, 2006

Region A (bioactive material) highlights the compositional range for bonding of bone to bioactive glasses and glass-ceramics. Compositions with greater than 60% SiO₂ do not bond and are bioinert (*region B*). In the *region C*, the reaction is too fast and there is not the formation of bonding the material is resorbable, while in the *region D*, the concentration of SiO₂ is too low, there is not the formation of the glass. *Region E*, in the middle of the Na₂O-CaO-SiO₂, typical of glasses with compositions constant 6 %wt of P₂O₅. In this region there are glasses with the highest level of bioactivity and rapid bone bonding (i.e. BG45S5). *Region S* points out the glasses with rapid reaction can form soft tissue bond. Once glass composition exceeds 52% by weight of SiO₂ the glass will bond to bone but not to soft tissues.

The bioactivity level of any material is measured by bioactivity Index (I_B). Bioactivity Index of a material is the time taken for more than half of the interface to bond. It is defined as follows:

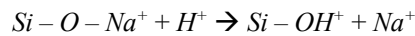
$$I_B = 100/t_{0.5}$$

BG 45S5 have I_B greater than 8, this means that it will bond to both soft and hard tissues. Instead, Materials such as synthetic hydroxyapatite with $0 < I_B < 8$ will bind only to hard tissue.

Bioglass mechanism of activity

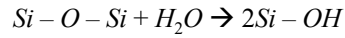
Bioglass 45S5 bonds with bone rapidly and also stimulates bone growth away from the bone–implant interface. The mechanism for bone bonding is attributed to a hydroxycarbonate apatite (HCA) layer on the surface of the glass, following initial glass dissolution. HCA is similar to bone mineral and is thought to interact with collagen fibrils to integrate (bond) with the host bone. Accumulation of dissolution products causes both the chemical composition and the pH of the solution to change, providing surface sites and a pH conducive to HCA nucleation. Five steps proposed for HCA formation can be observed in simulated simulated body fluid (SBF) *in vitro* or *in vivo* (J. R. Jones 2013):

1. Creating bonds Si-OH on the glass surface due to a rapid cation exchange of Na^+ and/or Ca^{2+} with H^+ from solution. Also phosphate is lost from the glass if present in the composition. So, the pH of the solution increases and a silica-rich region forms near the glass surface.



(Eq. 3.1)

2. High local pH leads to break of Si – O – Si bonds by OH⁻. Soluble silica is lost in the form of Si(OH)₄ to the solution, leaving more Si–OH at the glass–solution interface:



(Eq. 3.2)

3. Condensation of Si–OH groups near the glass surface: repolymerization of the silica-rich layer.
4. Migration of Ca²⁺ and PO₄³⁻ groups to the surface through the silica-rich layer and from the solution, forming a film rich in amorphous CaO–P₂O₅ on the silica-rich layer.
5. Incorporation of hydroxyls and carbonate from solution and crystallization of the CaO–P₂O₅ film to HCA.

Chapter 3

3 Composite scaffolds characterization

Polymeric scaffolds employed in tissue engineering and regenerative medicine exhibit certainly a quite complex structure. It is important to characterise these scaffolds not only qualitatively but even quantitatively, to determine parameters that relate to both the scaffold's suitability for sustaining cell growth and its structural properties (Morris, Mather, and Crowe 2009). The porosity, pore size distribution, degree of pore connectivity, structure "thickness" are just some of the important parameters to know in biomaterial design (Yang et al. 2014), (Conoscenti, Schneider, et al. 2017), (Lombardo et al. 2019). All those features could be evaluated separately by using various techniques that include scanning electron microscopy (SEM), gas-liquid porometry, mercury porosimetry, gas pycnometry (Ho and Hutmacher 2006). Moreover, in bone tissue engineering a number of techniques have been developed to fabricate porous scaffolds often modified or combined with bioactive fillers, for example bioactive glass (Dziadek et al. 2015), (Nikpour et al. 2018) or hydroxyapatite (HA) (B. Huang et al. 2018), (Türk et al. 2018), to achieve the desired mix of mechanical and biological properties mimicking those of a native human bone. In order to quantify the amount of filler present in a polymeric matrix a destructive technique known as Thermal Gravimetric Analysis (TGA) is frequently adopted (Azevedo and Reis 2003), (Z. Hong et al. 2004), (Blaker, Boccaccini, and Nazhat 2005). X-ray computed microtomography (micro-CT) is a non-

destructive tool that has demonstrated various key advantages and it is nowadays applied to different scientific or industrial fields such as biology (Shearer et al. 2016), material science (Salvo et al. 2003).

In this chapter, the innovative and traditional techniques used for the characterization of composite materials are described. Pure PLLA scaffolds were used as a control and reference in a several analyses.

3.1 Materials characterization

3.1.1 Micro-computed tomography (μ CT): Reconstruction and analysis

Over the past 30 years a rather new application of micro-CT concerns the bioengineering area (Otsuki et al. 2006), (Shearer et al. 2016). Several studies (Renghini et al. 2009) assessed the efficacy of micro-CT to investigate features of 3-D bioactive glass–ceramic scaffolds, whereas (Tuan and Hutmacher 2005) warns that the methods for evaluating osteo-integration of tissue engineered scaffold/cell constructs are based traditionally on 2D histological and radiographical techniques and only in rare cases on mechanical testing. With this respect, thanks to micro-CT technique, instead, it is possible to accurately characterise the bone growth into the scaffolds, to evaluate morphologically the 3D properties of scaffold/tissue composites during tissue regeneration and also to quantitatively assess osteoporosis progression and the effects of

aging on bone structure (Tuan and Hutmacher 2005). Employing of micro-CT technique makes the characterization of composite material simple and fast with the selection of appropriate parameter in the data processing step. Especially, with one single non-destructive analysis is possible to obtain a complete set of data on these complex polymeric structures. Furthermore, can be shedding light on any defects and imperfections that would cause problems in the regeneration of bone tissue, without damage to the construct.

Basic principles of tomography

The tool uses a point source that produces an X-ray cone beam whose geometry allows for optical magnification of the object on the detector system. By varying the distances between the X-ray source, the detector and the sample, the magnification can be set. However, during the acquisition phase, the distance of the sample from the source (SD) and the detector (DD) determines the pixel size (PS). Figure 3.1 shows the dataflow from 2D-image acquisition to 3D-image.

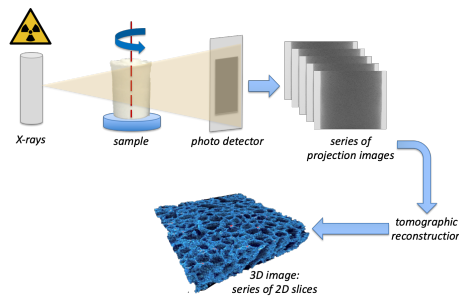


Figure 3.1 Schematic set-up of micro-CT analysis from the acquisition to 3D reconstruction.

In brief, X-rays crossing sample reach the detector providing a measure of the attenuation that radiation suffered along the path. The result of this process is the formation of a two-dimensional image called *projection images* obtained rotating the sample. The composition of these projections by mathematicians algorithms provides a *slice* (cross section image). In order to obtain the single slices at different heights, the rotation of the sample is combined with translation motion of the source-detector system. Using tomography principles, this series of images are reconstructed to produce a three-dimensional digital image where each voxel (volume element or 3D pixel) represents the X-ray absorption at that point.

Micro-CT the scanner employed for scaffold characterization was a Skyscan 1272 (Bruker), kindly made available by ATen Center, University of Palermo. In order to get the complete set of structural information, micro-CT analysis was carried out in 3 different steps by using Bruker's software:

1. Image Acquisition

The samples were placed on the rotating support and the images were obtained by setting a rotation step of 0.100° . The acquisition time was reduced by scanning only on half of a sample (rotation of 180°). In order to have an appropriate contrast for the reconstruction of polymer scaffolds we used an X-ray beam with an energy level of 40 kV and an intensity of 250 mA without filter. Images in TIFF format with a 16-bit colour depth were available at the end of the scans.

2. Image Reconstruction

3D-reconstruction and processing of data were carried out with software *N-Recon* (version 1.6.10.2 Bruker micro-CT, Kontich, Belgium) for elimination of artefacts and reduction of noise in each slice. The parameters of misalignment compensation, beam hardening, ring artifact reduction and smoothing have to set, in order to maintain a high definition of the image and at the same time correct the noise due to the acquisition process. These parameters are briefly described below:

- *Misalignment compensation*: it is useful to compensate the possible misalignments because a wrong alignment can create drag effects, double or blurry images.
- *Beam Hardening*: This artefact leads the object to appear denser near its surface and less dense in the central part. It is caused by different attenuation of X-ray arriving on the material surface. In fact, an incorrect value can cause the grey of the pixels to be altered, causing the density of a layer to be overestimated or underestimated. The parameter is minimized by the correct choice of the filter in the scan, since increasing the filter will decrease the beam hardening.
- *Ring artefact reduction*: This rebuilding phase occurs with the presence of concentric rings more or less marked and is much more accentuated the higher the resolution, but also depends in part on the density of the sample. This artefact can be minimized by carefully setting the exposure time in the scanning phase, choosing the appropriate filter and the tension of the X-ray beam.

- *Smoothing*: Smoothing is necessary to reduce the noise in the reconstructed image.

Setting appropriately these parameters, cross-section raw images in BMP format in greyscale were saved in 8 bits with 256 grey levels. Starting from the individual layers obtained, the different samples were visualized in 3D space thanks to *CT-Vox* software (version 2.7, Bruker micro-CT, Kontich, Belgium).

3. Extraction of quantitative parameters

All main quantitative parameters that allow one to gather information on scaffold's architecture were determined by *CT-Analyser (CTAn)* software (version 1.16.1, Bruker micro-CT, Kontich, Belgium). Quantitative analyses were performed in three separate portions of 300 layers indicated as Top, Middle and Bottom, respectively (Figure 3.2). For each of these, a "*Region of Interest*" (*ROI*) was chosen to further reduce processing time of 3D analysis. The ROI is a mask referring to the selected region (on a single cross-section image), which allows omitting what is outside the selected area from quantitative analysis. A "*Volume of Interest*" (*VOI*) was defined as the integration of all the ROIs across all the selected image levels, and defines the sub-volume of the dataset within which morphometric parameter calculation could be performed.

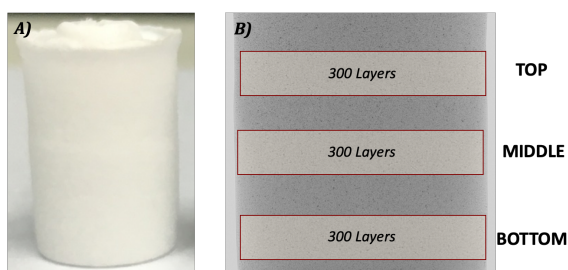


Figure 3.2 (A) Cylindric scaffolds as prepared by TIPS; (B) VOIs for quantitative analysis: Three regions of 300 layers, top, middle, bottom

Depending on the type of 3D analysis, raw images have been binarized by choosing an opportune threshold value. Greyscale threshold were determined comparing the scanning electron microscope (SEM) image with the μ CT pictures to highlight various details of scaffold. The images have been transformed from 8 bit to 1 bit. A fixed global threshold was chosen, which represented the transition in X-ray attenuation between less dense regions and denser. In this way separate analysis were carried out on polymer, filler and/or pores.

Following binary transformation, pixels with grey value lower than the threshold will become black whereas those with higher value will be white. This has allowed to highlight the objects (white pixels) subjected to analysis. The scaffold volume (SV) is obtained by multiplying the number of voxels corresponding to the X-ray absorption characteristics of the scaffold material by the volume of a single voxel. Parameters as Total Volume (TV), Object volume ($Obj. Vol$), Percent Object volume ($\% Obj. V$) can be

directly obtained from 3D analysis on the set of binary images. TV is the volume submitted for analysis related to the selected ROI and it is based on a simple count of the voxels contained in the volumetric model chosen. $Obj. Vol$ represents the part of VOI occupied by only white pixels. Finally, the ratio between $Obj. Vol$ and TV is called percent object volume. Also other important values such as structure thickness ($Tb.Th$) and objects number ($Tb.N$) are relevant to provide a thorough scaffold characterization (J. Kim et al. 2015). Structure thickness value was determined from software automatically, drawing diverse spheres inside the object and reporting only the value of sphere with the maximum diameter (Hildebrand and Ru 1997). Sample porosity (vol.%) could therefore be calculated as $(1 - SV/TV)$ in 3D space based on the void volume of the scaffold.

3.1.2 Scanning electron microscope (SEM)

Morphology of scaffolds was observed by Phenom Pro-X (Phenom-World, The Netherlands) (SEM). Each sample was brittle-fractured in liquid nitrogen and small pieces were fixed by conductive adhesive tape on aluminium stubs and sputter-coated with a gold layer in an argon atmosphere for 120 s under a current of 20 mA. A complete set of SEM images was taken with an electron beam energy of 10 keV and exported as 24-bit image files in .tiff format for further analysis.

The average pore size was attained by analysing several randomly selected portions of at least ten SEM images for each type of scaffold by using *imageJ* software.

3.1.3 Porosity evaluation

A gas pycnometer can be used for measuring the density or more accurately the volume of solids using method of gas displacement, by applying the pressure relationship known as Boyle's law (Haugen and Bertoldi 2017). As common, via this technique the real volume of a porous sample is estimated by excluding the volume of the pores. The porosity ε of the samples can be determined knowing the bulk volume (V_b) and volume of void calculated by pycnometer (V_v) thanks to the following equation:

$$\varepsilon = 1 - \left(\frac{V_v}{V_b} \right) \quad (\text{Eq. 5})$$

Here, the scaffold porosity was measured via gas pycnometer (Pycnomatic ATC, Thermo Fisher Scientific, Waltham, Massachusetts, USA), by adopting helium gas as penetrating agent. The dissection of specimens of cylindrical shape (15 mm diameter x 20 mm height) was needed in order to evaluate the bulk volume the samples.

3.1.4 Thermal gravimetric analysis (TGA)

In order to evaluate the amount of filler into the composite scaffold thermogravimetric analysis was carried out on each composite (at least in triplicate) by using a Labsys EVO, Setaram. After an initial stabilization, the temperature was increased from 30 °C to 500 °C at a constant rate of 10 °C/min under argon gas flow. The

final data were analysed and the results averaged to determine the residual mass with respect to the amount of filler (HA or BG) loaded.

3.1.5 Mechanical testing

Mechanical tests were performed using an Instron model 3365 (UK) mechanical tester equipped with a 1 kN load cell. The compression strength tests were carried out on cylindrical samples (diameter 13 mm, thickness 3 mm) applying at a crosshead speed of 1 mm/min. During test, the load was applied until to 80% strain of the porous samples started to occur. The elastic modulus (E) was calculated from the slope of the initial part of the engineering stress-strain curves.

3.1.6 XRD analysis

X-ray diffraction analysis (XRD) is one of the microstructural analysis methods. This nondestructive technique provides detailed information about the crystallographic structure, recognition of crystalline phases (polymorphism), chemical composition, and physical properties of materials (Inan 2017). The incoming X-ray beam can be characterized as a plane wave of radiation interacting with the electrons of the material under study. The interaction is in the form of both absorption and scattering. In a crystalline material, the scattered X-rays undergo constructive and destructive interference. The diffraction of X-rays by crystals is described by Bragg's Law:

$$n\lambda = 2d \sin\theta$$

(Eq. 6)

Where n is a positive integer, λ is the wavelength of incident wave, d is the distance between two crystalline plane and θ is the scattering angle. The directions of possible diffractions depend on the size and shape of the unit cell of the material. The intensities of the diffracted waves depend on the kind and arrangement of atoms in the crystal structure.

XRD measurements were carried out using a benchtop X-ray diffractometer (Miniflex600). The data were recorded in the range of 10° - 70° with step at $2\theta = 0.01^\circ$, scanning time of 15 s and Cu-K α -radiation generated at 40 kV and 15 mA.

3.1.7 FTIR analysis

FTIR spectroscopy is the most widely used technique for biomaterials characterization to elucidate the molecular structure. When biomaterials are irradiated by FTIR light, absorption occurs if the IR frequency coincides with the vibrational frequency of a bond present in biomaterials. The absorbance frequencies are characteristic of the chemical groups and their chemical environment (Pradhan et al. 2017).

Here, attenuated total Reflectance-Fourier Transform Infrared spectroscopy (ATR-FTIR) technique was conducted by Shimadzu IRAffinity-1S. The spectra were acquired in the

range of 400 - 4000 cm^{-1} wave number by averaging 40 scans at the standard resolution of 4 cm^{-1} .

3.1.8 In vitro bioactivity study

Scaffold bioactivity was evaluated by examining the ability of apatite (HCA) to form on its surface in a simulated body fluid (SBF), according to standard protocol proposed by Kokubo and Takadama 2006. SBF is a fluid with ion concentrations nearly equal to those of human blood plasma (Table 3.1).

Table 3.1 Ions concentrations of SBFs and human blood plasma

	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}
Human blood plasma	142.0	5.0	1.5	2.5	103.0	27.0	1.0	0.5
SBF	142.0	5.0	1.5	2.5	148.8	4.2	1.0	0

Briefly, SBF was prepared adding gradually in 700 ml of distillate water the following reagent: NaCl, NaHCO_3 , KCl, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, CaCl_2 and Na_2SO_4 . The solution in a plastic beaker was maintained under stirring and at temperature of 36.5 ± 1.5 °C. Tris-hydroxymethyl aminomethane ($(\text{HOCH}_2)_3\text{CNH}_2$) (Tris) was added as the last reagent and drops of 1.0 m-HCl were used to raise the pH value to 7.4. After cooling, the final volume was made up to 1000 ml, the solution was stored in the fridge at

5-10 °C and was use within 30 days from preparation. Samples were soaked in a volume of SBF different depending on the sample volume.

Afterwards, the specimens were incubated at 37 °C in an orbital shaker incubator (105 rpm) for different times. The solution was refreshed every 3 days in order to restore the initial ions concentrations because at pH lower than 8 calcium phosphate precipitation was not observed (Cerruti, Greenspan, and Powers 2005; Vollenweider et al. 2007). For each time point, three samples were recollected and gently rinsed with deionized water and subsequently with acetone to terminate any reaction. Then, they were dried at room temperature for 48 h. Furthermore, SEM observations, XRD and FTIR spectrometry were performed to analyse respectively the morphology, crystallographic and chemical structure of formed apatite layer (HCA) after soaking in SBF.

3.2 Biological characterization

3.2.1 Live/dead cell viability assay

Live/dead assay is a staining test used to test cell viability by labelling alive and dead cells with different fluorescent dyes in a unique, fast and simple assay protocol (Stoddart 2011). The staining solution for live/dead assay was prepared: 1 ml of PBS was mixed with 5 µl of fluorescein diacetate (FDA) solution (5 mg/ml in acetone) and 1 µl of propidium iodide (PI) solution (2 mg/ml in PBS). The first dye, FDA is a non-fluorescent molecule which permeates cell membrane, reacts with intracellular enzymes and becomes

fluorescent labeling the living cell green. Instead, PI enters in contact with cells having damaged plasma membrane and labels them with a red color the dead cells. It is not able to permeate an intact plasma membrane and strongly binds to cell DNA. A Leica Confocal Laser Scanning Microscope (CLSM) was used to observe samples and Leica LasX software was adopted to take pictures.

3.2.2 Haematoxylin/Eosin histologic assay

Haematoxylin/eosin staining technique is a widely used method in histology to observe any kind of tissue; it is usually performed in transversal sample's sections deposited in glass slides and, after the assay, the stained tissue can be easily observed with a light microscope. Haematoxylin is a salt which, in contact with water, dissociates in ions: its positive-charged molecules tend to bind to phosphate groups of nucleic acids, labelling them blue or dark purple. Because cell nuclei contain high amounts of nucleic acid situated in DNA and RNA, during the observation they appear with these colors. Like haematoxylin, also eosin is a salt soluble in water; its negative ions combine with cytoplasmic proteins, making them of a color that ranges from red to pink. Eosin is able to stain collagen fibres as well (Yeatts and Fisher 2011).

3.2.3 Vitality test

The assay methods allow to evaluate cell viability by detecting the metabolic activity of cells. In this work, the number of viable cells was estimated by using one of the reagents listed below:

- *Cell counting Kit 8* (Sigma Aldrich), a sensitive colorimetric kit containing *WST-8*, a salt reduced by mitochondrial dehydrogenases to orange formazan (Feng et al. 2015). The amount of formazan produced is directly proportional to the number of living cells and can be estimated in absorbance at 450 nm (Chamchoy et al. 2019; Feng et al. 2015).
- *Alamar Blue test* uses a blue-coloured fluorescent/colorimetric dye called resazurin: it is a redox indicator that emits fluorescence and changes colour (turns pink) when it is reduced to resorufine by enzymes produced by metabolically active cells that proliferate. The cell number was determined in fluorescence ($\lambda_{\text{excitation}} = 530 \text{ nm}$, $\lambda_{\text{emission}} = 590 \text{ nm}$).

The viability test was performed incubating cells or scaffolds with 500 μl of a solution 1:10 v/v dye reagent diluted in fresh for 3 h at 37 °C and 5% CO_2 . After that, the medium was collected in aliquots of 100 μl and transferred to a 96-well plate for reading optical density by spectrophotometric plate reader. The cell number on each specimen was calculated based on a standard curve prepared with predetermined cell numbers.

3.2.4 Statistical analysis

All quantitative results were obtained from at least triplicate samples. Data of different tests were expressed as mean values with standard deviation. Statistical significance in this study was evaluated using a one-way ANOVA with a post hoc Bonferroni and Tukey test. Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.001$. All analyses were carried out using Origin 2018 software.

Chapter 4

4 Composite scaffold PLLA-HA

Biomimetic composite scaffolds with a structural and chemical composition similar to native bone tissue may be promising for bone tissue regeneration.

In this chapter, PLLA-HA scaffolds produced via TIPS are proposed as substitute to repair the bone tissue. HA is an excellent biocompatible biomaterial, which is also bioactive, osteoconductive, nontoxic and non-inflammatory agent. Osteo-conduction makes HA a suitable material for hard tissue replacements.

Composite scaffolds were produced at the University of Palermo but biological characterization of PLLA/HA samples was carried out in the laboratories of Abiel s.r.l.

4.1 PLLA-HA scaffolds

PLLA-HA scaffolds were fabricated via TIPS starting from a homogenous polymer solution adding HA particles. A solution of poly-L-lactic-acid, 1,4 dioxane and distilled water (polymer-solvent-nonsolvent) was prepared with a PLLA concentration of 4% wt/wt and ratio solvent/nonsolvent of 87/13 wt/wt. Different HA amounts (0, 10, 20, and 50%) were dispersed in the ternary solution. In the first study, HA microspheres were a kind gift by Professor Licciulli from University of Salento and produced via spray drying

(Cholas et al. 2016). In the other one, HA was produced in situ via wet chemical method (Eslami et al. 2010).

4.1.1 Scaffold preparation

The suspension made of a homogeneous ternary solution and HA particles was prepared following the thermal protocol designed by Carfi Pavia et al. 2018. The suspension was initially kept at 60 °C and sonicated for 30 minutes at 35 kHz, in order to improve the filler dispersion and thus minimize the formation of macro-aggregates. Scaffolds were obtained by hot pouring the solution into a High-Density Poly-Ethylene (HDPE) cylindrical holder sample (inner diameter 17,6 mm and height 35,7 mm). The molds were first immersed into a thermal bath at 30°C, the temperature within the metastable region, for 15 minutes. Later, a quench by direct pool immersion in an ethyl alcohol bath at a temperature of −25 °C was performed (Figure 4.1). The foams obtained were subjected to washing in deionised water and drying at 35 °C under vacuum, in order to completely remove any remaining solvent trace.

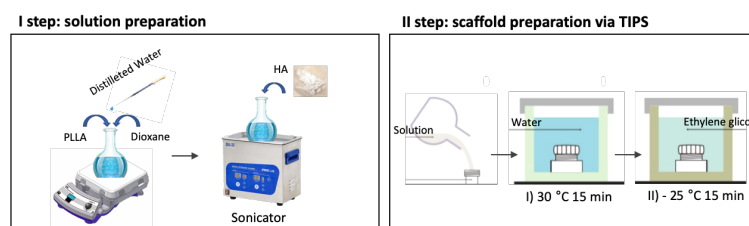


Figure 4.1 Steps of scaffold preparation via TIPS: I) Preparation of ternary solution and adding of HA (filler); II) thermal path to obtained scaffold via TIPS

4.1.2 Scaffold characterisation

Bulk morphology and morphological parameters of PLLA-HA scaffold were examined comparing traditional and innovative investigation methods. Micro-CT was proposed as innovative technique to determine porosity, average pore size, HA volume amount and spatial distribution of complex porous structures with one single non-destructive analysis. In order to assess the reliability and robustness of the micro-CT approach, extracted results were compared with the values obtained by traditional destructive techniques (i.e. SEM, TGA, gas pycnometer), as previously illustrated in the 3.1 paragraph. In this specific case of TGA measurements, composite scaffolds with mass of about 5 mg and neat PLLA of 3.5 mg were used.

Through microcomputed tomography, a thorough assessment of three-dimensional structure of scaffolds, including morphological details was carried out. Images with two different pixels sizes (PS): 3.75 μm (PS1) and 1.25 μm (PS2) were processed. As well known, the voxel value is strictly linked to PS, thus PS1 and PS2 correspond to a voxel

volume of $53 \mu\text{m}^3$ and $2 \mu\text{m}^3$, respectively. By reconstructing the complete set of projections, the images of the so-called "reconstructed cross section" are provided (Figure 4.2). The fundamental parameters, such as misalignment, rings artefact, beam hardening, smoothing were set manually through a trial and error procedure, in order to maintain a high definition of the image and reduce artefacts related to the acquisition process. Post-alignment value for neat PLLA scaffold has been chosen equal to 5, while it has been set within a range 8.5-9.5 for all the others. As a matter of fact, upon increasing filler concentration it was necessary to set higher values accordingly. For all the other parameters, the values were kept constant also for the composite scaffolds. An example of three-dimensional reconstructions with different pixel size was reported in Figure 4.3 for PLLA/HA 90/10 wt/wt scaffolds.

All quantitative parameters were determined according to the protocol proposed in 3.1.1 paragraph. For each portion of 300 layers, a ROI of square shape $10^4 \times 10^4 \mu\text{m}$ was chosen and a parallelepiped *VOI* of 8 mm^3 was defined. Depending on the type of 3D analysis, raw images have been binarized by choosing an opportune threshold value. Different threshold values (Table 4.1) were selected to carry out the quantitative analysis regarding polymer, amount of HA and pores size independently. The respective images were reported in Figure 4.4. In order to be sure of the threshold values chosen, the images so obtained were compared with SEM micrographs, trying to obtain a good compromise between the grayscale images and the binary ones.

The average particles size was calculated in the 3D space as the structure thickness that best approximates the diameter of HA particles.

A different protocol was followed to estimate the average pores size. Pores analysis was conducted in three different 2D-slices for each VOIs. In brief, binary slices were obtained with opportune threshold values to highlight in white the pores. A despeckle filter was applied to remove any pixels recognized as artefacts and the pores were displayed about like circles with solid line. For each pore, the circle diameter of equivalent area (EDCa) was analysed. The average pores size in each specimen was identified from the distribution of percentage of objects with specific EDCa vs. the dimension range of objects. Comparing the raw and binary images only pores with EDCa in the range between 50 – 150 μm were considered. Indeed, objects with EDCa < 50 μm were identified as artefacts and objects with EDCa > 150 μm as coalescent pores. Finally, in order to estimate the average pore diameter value, the ratio between all the objects having diameter in the range analysed and the total number of objects (pores) present in the layer was calculated.

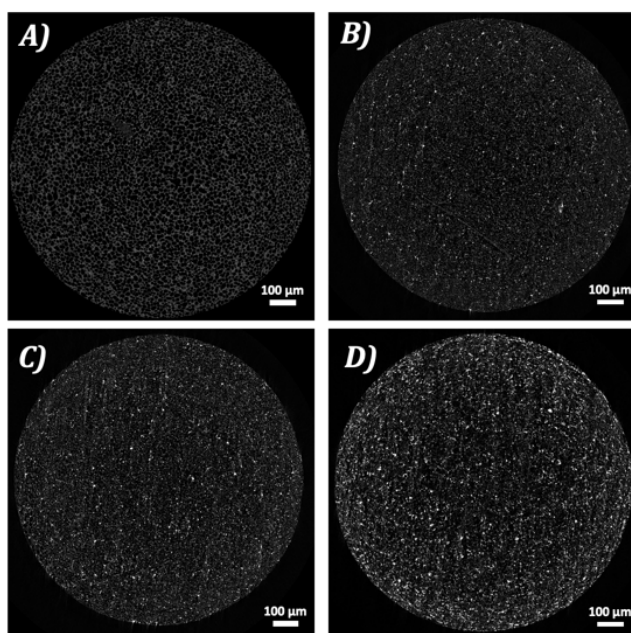


Figure 4.2: Images of cross section reconstructed (PSI) of different type of scaffolds: A) neat PLLA; B) PLLA/HA 90/10 wt/wt; C) PLLA/HA 80/20 wt/wt; D) PLLA/HA 50/50 wt/wt

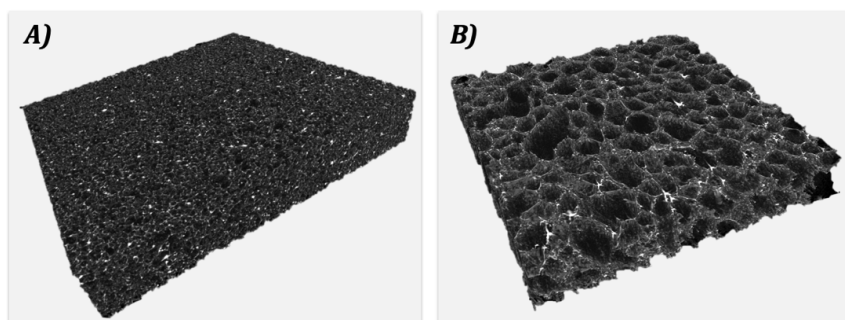


Figure 4.3 3D reconstruction of composite scaffold PLLA/HA 90/10 wt/wt: A) image pixel size of 3.75 μm (PS1); B) image pixel size of 1.25 μm (PS2)

Table 4.1 Thresholding values used for images binarization depending on the typology of quantitative analysis

THRESHOLDING VALUES

HA %wt	Pores	Polymer	Hydroxyapatite
0	0 - 8	8 - 128	-
10	0 - 30	30 - 160	160 - 254
20	0 - 35	35 - 166	166 - 254
50	0 - 30	30 - 110	110 - 254

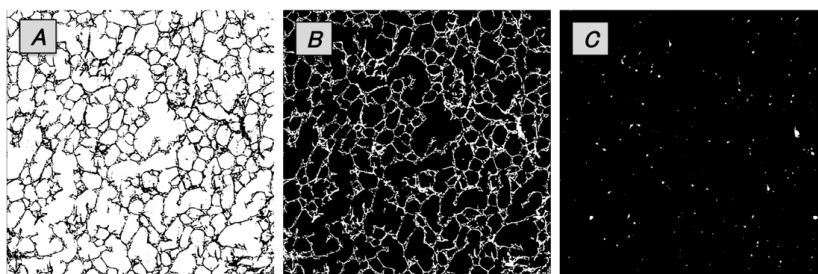


Figure 4.4 Threshold range values with related binarized images for: A) interconnected pores (0-30); B) PLLA polymer mesh of scaffold (30-160); C) HA particles (160-254). Example of scaffold PLLA/HA 90/10 wt/wt

4.1.3 Results

Cylindrical polymeric scaffolds with 0, 10, 20, 50 % wt HA concentrations (diameter 14,5 mm, height 21,6 mm) produced via TIPS and their inner structure and morphological parameters were determined by micro-CT analysis. The protocols proposed for each single image analysis are validated by comparing micro-CT results with traditional methods.

Porosity evaluation

The porosity calculated by micro-CT can be evaluated on the void volume of the composite. Porosity values measured by gas pycnometer and micro-CT using pictures with different pixel size were shown in Table 4.2. It easy to observed how values derived by micro-CT are not completely in agreement with the value of bulk porosity, but slightly underestimated. This difference was even more pronounced when the image analysis was

carried out on pictures with a higher pixel size (PS1), because in this case the pixels of background are not correctly identifiable and the amount of polymer is overestimated in the binarization step. According to other researches (Hui-Yan et al. 2005; Moore et al. 2004; Freire-Gormaly et al. 2015), the comparison of the porosity values arising from 3D analysis shows that this value is strongly dependent on the imaging pixel size and on threshold values.

Table 4.2 Porosity of composite scaffolds at different PLLA/HA weight ratios by different methods. Mean value was evaluated between sections of Top, Middle and Bottom and then standard deviation (SD) was calculated

	GAS PYCNOMETER		MICRO-CT PS1		MICRO-CT PS2	
HA %wt	Mean value	SD	Mean value	SD	Mean value	SD
0	91.5	0.1	76.7	0.42	88.2	0.42
10	95.3	0.75	78.99	0.16	87	0.20
20	94.7	0.25	71.97	0.51	91.2	0.21
50	92.9	0.23	70.35	0.68	86.94	0.64

Average pores size

Representative SEM micrographs of neat PLLA and PLLA/HA scaffolds at different polymer/filler ratios are shown in Figure 4.5. The pictures reveal a continuous structure with 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. Similarly, to SEM images, the three-dimensional viewing reveals a homogeneous HA distribution and HA particles can be distinguished from the polymer matrix for their higher density (Figure 4.3). SEM can provide information about the pore size but the retrieved informations are limited to a 2D plane, therefore the volumetric pore connectivity cannot be determined.

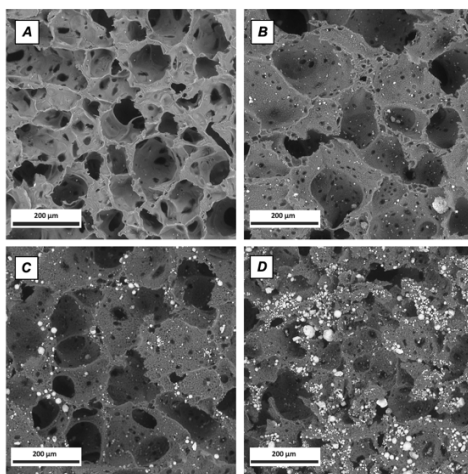


Figure 4.5 SEM micrographs of (A) neat PLLA scaffold; (B) 90/10 wt/wt PLLA/HA scaffold; (C) 80/20 wt/wt PLLA/HA scaffold; (D) 50/50 wt/wt PLLA/HA scaffold.

In Table 4.3, average pore size values estimated from SEM images are compared with the values obtained by micro-CT slices at the two PS. Average pores size was then evaluated through 2D model method on 3 different layers for each VOI. As known, a

closed pore in 2D (surface of black pixels completely surrounded with white pixels) could be extended in the 3D space by connecting to each other upper and lower slices. In this case, the morphology of the sample is characterized by a high level of interconnection. This feature leads to a not-resolvable binarization process, since it is not possible to discriminate in 3D every single closed pore without influencing its final diameter. To overcome this problem, it was decided to show the pores in 2D as objects (white pixels) in order to correlate the EDCa to pore diameter. As for the estimation of porosity, also in this case the values achieved with PS2 are closer to those obtained from SEM micrographs with respect to those obtained with PS1.

Table 4.3 Average pore size [μm] of scaffold with 0, 10, 20, 50 %wt HA. Comparison of values obtained via different techniques.

	<i>SEM</i>	<i>MICRO-CT PS1</i>	<i>MICRO-CT PS2</i>
<i>HA %wt</i>	<i>Average value [μm]</i>		
0	103	90.2	101.2
10	127	95.54	109.8
20	127	90.51	110.4
50	123	89.8	110.9

HA content

In order to retrieve the HA content via micro-CT image analysis two analysis with different threshold values were carried out on the same dataset of images. First, an

appropriate threshold value in each layer (Figure 4.4 B) was chosen to estimate the PLLA percentage in different samples. A second 3D analysis was carried out to light up only particles (Figure 4.4 C) and % Obj. V was determined. Through analysis of HA particles, we observed that the amount of fillers (% Obj.V) in the three sections is approximately constant so particles sedimentation does not take place-during the scaffold preparation process (Figure 4.6). These results highlight the validity of the TIPS technique for the production of composite scaffold from low (10% wt) to relatively high (50% wt) filler concentrations. The combination of particle and polymer analyses data allowed the quantification of the HA weight percentage in all samples. The density values used to convert v/v to w/w are 3.16 g cm^{-3} and 1.25 g cm^{-3} , respectively, for HA and for the polymer. According to the data shown in Figure 4.7 the weight percentage of HA increases linearly with a trend in agreement with the nominal value, even though the amount of HA calculated by 3D analysis is slightly underestimated with respect to the nominal value. This result, confirmed by TGA test, demonstrates the reliability of the 3D quantitative analysis for samples with 10 and 20% wt of HA. Moreover, the study shows how the choice of PS is relevant for the investigation of porosity but it does not influence the final result for the estimate of filler amount.

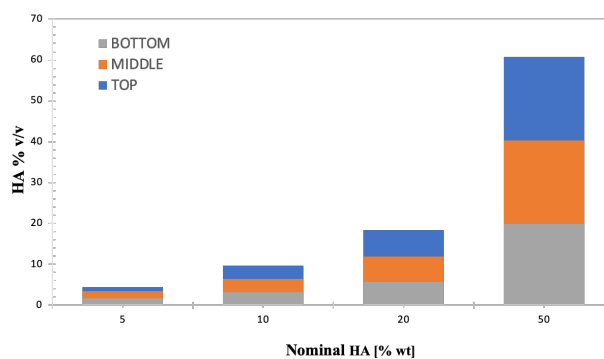


Figure 4.6 HA quantitative analysis by micro-CT for all composite scaffolds in each VOIs. HA values (%v/v) are reported as a function of nominal HA (%wt).

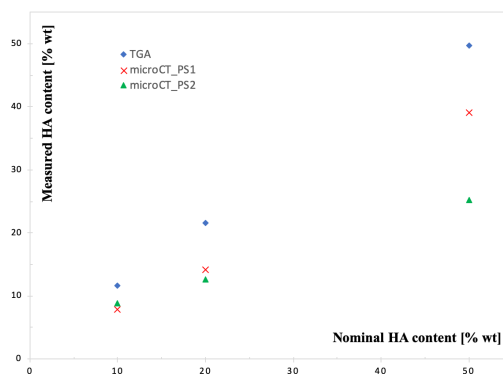


Figure 4.7 HA weight percentage determined by thermal gravimetric analysis (blue rhombus), micro-CT_PS1 (red x) and micro-CT_PS2 (green triangle).

Furthermore, the particle size distribution was evaluated by reporting the value of the structure thickness returned at the end of the HA analysis. The hydroxyapatite particles in all samples exhibit a mean size in the range of 11 to 20 μm (Figure 4.8 A). These data are in agreement with those obtained from the light scattering granulometer analysis on HA particles (Figure 4.8 B) showing that a good level of dispersion has been obtained.

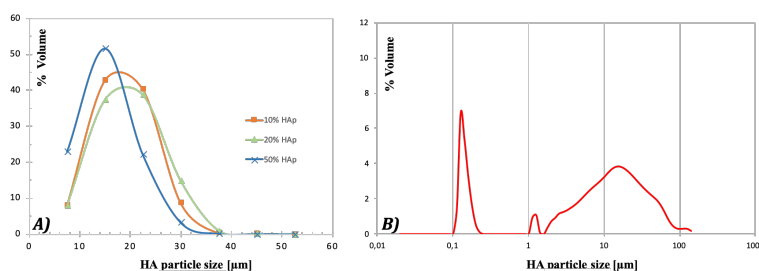


Figure 4.8 (A) HA particles size distribution in composite scaffolds with different HA weight percentages: 10 %wt (orange), 20 %wt (green) and 50 %wt (blue); (B) HA particles size [μm] obtained by granulometry analysis.

Micro-CT, in spite of being a rather new technique, has demonstrated numerous key advantages related to its peculiar nature of computational method that allows the calculation of numerous parameters directly on 3D space. On the other hand, several drawbacks must be taken under consideration, among which the arise of artefact during the acquisition step (beam hardening, misalignment) and the difficulty to set the thresholding values for the distinct quantitative analysis, especially in specimens containing a filler. The issue is more pronounced when extremely complex morphologies

are encountered, as in the case study reported, where the samples are characterized by a composite structure with interconnected pores and a high level of porosity.

Even though its main limit is linked to the pixel size of pictures (especially for porosity, pore size and filler size analysis), micro-CT imaging may be used to provide a complete set of data on structural characteristics of samples. Therefore, micro-CT analysis could be successfully employed as a non-destructively technique both for the quality control and for prediction of performance before scaffold implantation for in *in-vivo* tests.

4.2. Osteoblast differentiation in 2D culture

Before to investigate the osteoblast proliferation and differentiation on the three-dimensional scaffold, a preliminary study of osteoblasts behavior cultured in 2D under specific condition was studied. Under certain stimuli, osteoprogenitor cells may differentiate into premature osteoblasts and further into mature osteoblasts. Here, this process was detected by increased alkaline phosphatase (ALP) activity.

4.2.1 In vitro cell culture

Mouse pre-osteoblastic MC3T3-E1 (Sigma Aldrich) cells were cultured in a proliferation medium in 75 cm² cell culture flasks incubating at 37 °C with 5% CO₂. The medium was prepared with Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich) supplemented with 10% of fetal bovine serum, 1% of glutamine and 1% of streptomycin/penicillin. Upon 80% confluency, cells were detached with the standard protocol using trypsin/EDTA solution and counted through a Burker chamber. A density of 6000 cells/cm² was seeded in 12-well plates with 1 ml of complete medium. After 7 days, all wells were divided into osteoblast differentiation (OS) group and control (CON) group. The cells of OS group were grown in osteoblast differentiation medium (labeled osteo-inductive medium) containing basic culture medium supplemented with 1 µM dexamethasone, 10 mM β-glycerophosphate, and 50 µg/ml ascorbic acid in 5% CO₂ at 37 °C, while the cells of CON group were grown in only proliferation medium. MC3T3-E1 cells were cultured until 28 days and both viability test and ALP activity assay were performed. For each time three samples were analyzed (n=3).

4.2.2 Cell proliferation and differentiation

Cell numbers on different wells were measured using the Alamar Blue assay on days 1, 7, 14, 21 and 28, as illustrated in 3.2.3 paragraph. The results were reported as relative cell viabilities obtained by dividing the cell number on each well by that initial at 1 day from seeding.

Cell differentiation ability in different culture medium was evaluated by measuring the activity of alkaline phosphatase (ALP) in cell lysates using a SensoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec) following the manufacturer's instruction. In particular, incubating the protein extracts with the enzymatic substrate, p-nitrophenyl phosphate (pNPP), a colorimetric reaction caused by dephosphorylation occurs, bringing to the formation of a yellow product, p-nitrophenol. In order to obtain protein extract of MC3T3-E1, the pellets obtained from the three wells was collected in one Eppendorf tube and lysed with 80 μ l of 0.2% Triton X-100 at 4 °C with speed of 2500 g for 15 min. ALP activity was recorded as the amount of nitrophenol released through the enzymatic reaction, and the absorbance was measured at 405 nm. ALP activity was reported as relative percentage ALP activity, which was obtained by dividing the ALP produced in osteo-inductive medium by that in control medium at 7, 14, 21, 28 days. As initial value for the measurements the ALP activities was chosen the day 7, before the changing the control medium with osteo-inductive.

4.2.3 Results

The proliferation of MC3T3-E1 cells cultured with cell culture medium control and osteo-inductive was studied by examining cell viability on 1, 7, 14, 21, 28 days. The relative cell viability was calculated in both cases and the trend is illustrated in Figure 4.9. The number of cells on all well increases with the culture time. Until 14 days, there is not change in cell proliferation among two groups, the cells show a same behaviour regardless of the cell culture medium adopted, which may be explained by growth inhibition by over-confluent cells in the well-plate, according to D. Hong et al. 2010. A significant difference ($p < 0.01$) between the two groups of cells cultured with OS and CON medium after 21, 28 days is evident. This result confirms that combination of ascorbic acid and dexamethasone promotes osteogenic ability of MC3T3-E1 cells reducing the proliferation (Langenbach and Handschel 2013; Yan et al. 2014).

To evaluate the effect of cell culture medium composition on the osteogenic capacity of MC3T3-E1 cells, the ALP activity at 7, 14, 21, 28 days was evaluated. In Figure 4.10, the ratio percentage of ALP activity of MC3T3-E1 cells in OS and CON group is displayed. An increasing of ratio percentage of ALP is evident between 14 and 21 days, suggesting a decreased cell proliferation at the step of osteoblast differentiation (Peng, Yu, and Wei 2011). According to D. Hong et al. 2010; Quarles et al. 1992, the different steps of osteoprogenitor cell maturation in the MC3T3-E1 cells can be identified. In the first step (7 - 14 days), the MC3T3-E1 cells showed and increasing slightly of ALP ratio and take place the early stage of osteoblast differentiation. In the second step (14 - 21 days), the MC3T3-E1 cells demonstrated increased ALP activities and osteoblast differentiation is

evident. This is a transformation period where premature osteoblasts turn into mature osteoblasts. After 21 days, the ALP ratio decreases because of further increasing of cell proliferation.

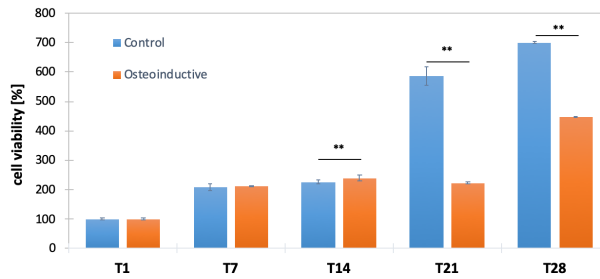


Figure 4.9 Percentage relative cell viability of MC3T3-E1 cells cultured in two different cell culture medium: control and osteo-inductive medium, respectively. Effect of different cell culture medium was investigated. Error bars represent means $\pm$ SD for $n = 3$. Asterisks denoted a significant difference between two groups, $**p < 0.01$.

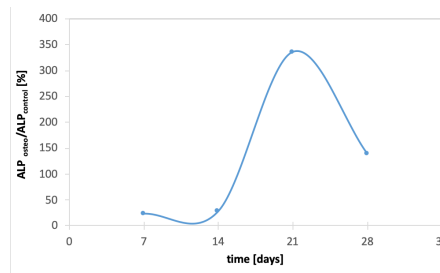


Figure 4.10 Percentage change in the ratio of cell ALP activities of MC3T3-E1 cultured in osteo-inductive/control medium.

Summarizing, MC3T3-E1 cells cultured in osteoblast differentiation medium at 7 day were still in an undifferentiated status and cells treated for more than 14 days were considered as mature osteoblasts. Therefore, these osteoprogenitor cells under osteoblast differentiation between 7-14 days were in the early stage of osteoblast differentiation. It is a critical stage of transformation from premature osteoblasts to mature osteoblasts.

4.3 Osteoblasts in composite PLLA/HA scaffolds

4.3.1 Scaffolds preparation and characterization

Two different HA concentration, 5% and 7% wt, were added to solution 4% wt PLLA following the steps described above (Figure 4.1).

Hydroxyapatite produced via sol-gel was bought by CamCeram® III (Alhenia AG, Dättwil, Switzerland). First of all, HA particles size were crushed into an electric rotating grinder (A10 basic, IKA®) at the speed of 25000 rpm and the particles size distribution was investigated by a laser diffraction technique through a Mastersizer 2000 (Malvern Instruments Ltd., Royston, United Kingdom). To analyze the particles size, a laser diffraction technique was carried out through a Mastersizer 2000 (Malvern Instruments Ltd., Royston, United Kingdom) in order to evaluate the particle size distribution. Afterwards, 0.1 g of nHA particles were dispersed in 0.8 L of distilled water and the average of 10 measures was reported.

Scaffolds PLLA/HA wt/wt ratio of 95/5, 93/7 were obtained maintaining the solution in a thermal bath at 30°C for 30 minutes, where take place the TIPS process. After that, the

final structures were locked in the bath at -25 °C. Two different HA concentrations were tested in order to study the effect of the filler amount on the osteoblast proliferation and differentiation.

Micro-CT analysis was performed to characterize the samples in a non-destructive way, preserving the morphology of the scaffolds. By setting the scanning parameters as described before (3.1.1 paragraph), images with a pixel size of 3.75 µm were obtained with a scan duration of nearly 4 h for every sample. All images so obtained were reconstructed and displayed in the 3D space.

4.3.2 In vitro cell culture

The cytocompatibility of composite scaffold was assessed by *in vitro* cell tests in terms of attachment and proliferation of MC3T3-E1 pre-osteoblastic cells. MC3T3-E1 cells were cultured in a complete medium as reported above. PLLA/HA scaffolds were reduced to circular-shaped samples (diameter: 5 mm, thickness 1 mm) and were sterilized under vacuum in a 70% ethanol solution. Afterwards, scaffolds were abundantly rinsed in sterile PBS 1× for 2 h and then treated with Micro-fibrillary rat-tail type-I collagen (BD Biosciences) in 0.02 N acetic acid at the concentration of 100 µg/ml for 90 min. Finally, the scaffolds were washed in complete medium to activate the collagen mesh formation thanks to neutral pH shift. Thereafter, 25 µl of cellular suspension at the concentration of 10⁴ cells/µl were kindly inoculated into each scaffold. After an incubation at 37°C and 5% CO₂ for 90 min to promote cell adhesion, each scaffold was transferred into a well of a 24 well plate and fresh medium was added. The culture medium was replaced every three

days up to 49 days in order to maintain constant the pH and the nutrients level for the whole experiment.

4.3.4 Cell viability assay

Cell proliferation on scaffold of PLLA/HA %wt ratio of 95/5, 93/7 was evaluated through an established protocol with Cell counting Kit 8 (3.2.3 paragraph) taking care to transfer the scaffolds into new wells to avoid the quantification of cells adhered to plate surface. The assay was carried out at 1, 7, 14, 21, 28 and 49 days of culture in triplicate ($n = 3$) for each time. Not seeded scaffolds were used as negative controls in each measurement. Cells amount on the scaffold after 1 days was taken as reference and it was normalized to 100%.

4.3.5 Fluorescence microscope

Scaffolds, after a culture in the incubator for 0, 7, 14, 21, 28, 35 and 49 days were put into a 24-well polystyrene plate and washed 4 times with complete 1X PBS. Then cells were fixed in 10% formaldehyde in milli-Q water for 15 min at room temperature and finally the scaffolds were washed twice in the 1X PBS. After the washings, all nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Merck KGaA) for 30s at room temperature. Images were taken under a fluorescence microscope (Leica DM 2500, Leica Microsystems) equipped with a charge-coupled device camera.

4.3.6 Alkaline phosphatase activity

The osteoblastic differentiation process was investigated as reported above, measuring the alkaline phosphatase activity, a marker expressed during osteoblastic

maturation. According to the protocol describe in the paragraph 4.2.2, the samples were incubated with 100 μ l of 0.2% Triton X-100 lysis buffer to obtained the extracts containing alkaline phosphatase. The measurements were performed only for PLLA-HA 7% wt at 7, 21, 28 and 49 days in triplicate. Data are expressed in ng/cell using a standard curve to convert absorbance values in ng of alkaline phosphatase.

4.3.7 Results

Figure 4.11 shows that the initial average particle size of HA powder was in the range of 10-100 μ m. After grinding treatment, the dimensions of filler particles were significantly reduced the ranges from 0.1 to 4.0 μ m. As reported by Domingos et al. 2017, dimension of HA particles influences the cell adhesion and proliferation. In particular, composite scaffolds filled with nano-HA presented higher cell viability when compared to micro-HA.

SEM images (Figure 4.12 A, B) exhibit the porous structure and morphology of composite PLLA/HA scaffolds obtained via TIPS. Regardless of HA concentration, the samples appear to have a homogeneous structure with interconnected pores with an average pore size of 100-150 μ m. Through a μ CT scanning it was possible to display the structure of composite scaffolds in 3D space. Three-dimensional image of PLLA-HA 7% wt, visualized in Figure 4.13, was reported as example. These pictures confirm a morphology characterized by open and well interconnected pores with a homogeneous distribution of particles inside the entire polymeric mesh.

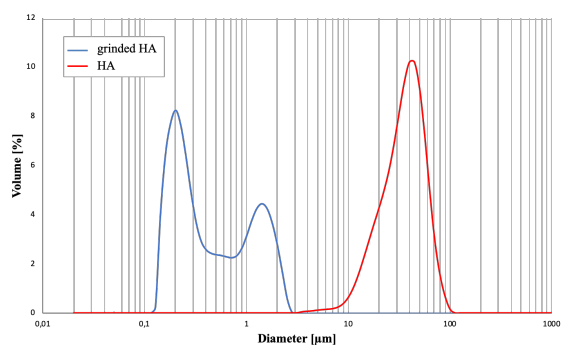


Figure 4.11 Comparison between particle size distribution of HA before (red line) and after (blue line) grinding treatment

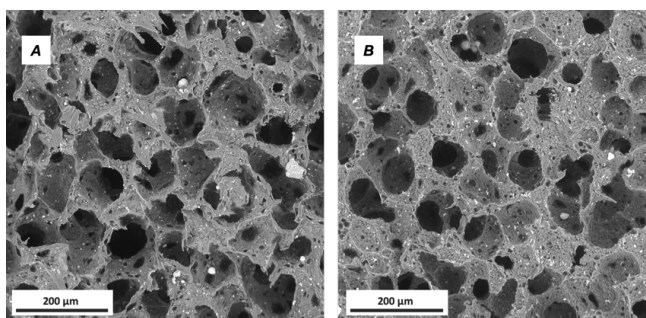


Figure 4.12 SEM micrographs of PLLA-HA scaffolds produced via TIPS keeping the demixing temperature at 30 °C for 30 min: (A) weight ratio of 95/5, B) weight ratio of 93/7.

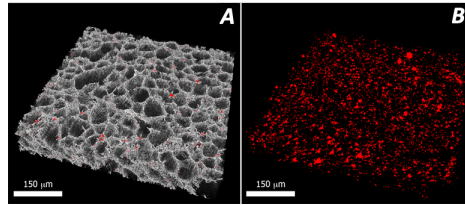


Figure 4.13 μ CT reconstruction of PLLA-HA 7 %wt scaffold. (A) 3D volume visualization by CTVox software of whole scaffold, (B) HA distribution inside this volume. Polymer is highlighted in grey and particles in red

In this way, the PLLA/HA scaffold obtained by TIPS methodology ensured a structure suitable for cell seeding, colonization and migration (Carfi Pavia et al. 2018; Conoscenti et al. 2017). The relevance of achieved scaffold pore size was under- lined recently by Seong et al., 2017, who verified that pore size of $\pm 100 \mu\text{m}$ enhances both cell-cell and cell-ECM interactions because of the large surface area, facilitating cell migration and proliferation.

Cell viability percentage as a function of days of culture on both samples, is reported in

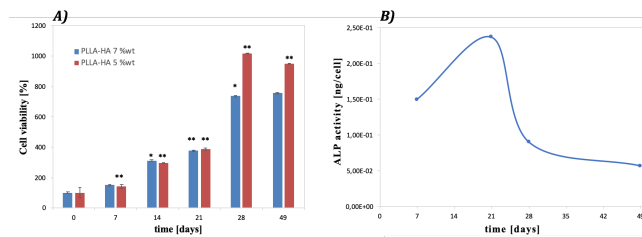


Figure 4.14 A. From the whole culture time taken in consideration for PLLA-HA 5 %wt, viable cell number had regularly grown, reaching the maximum significant peak at 28 days ($p < 0.001$). Between 28 and 49 days a significant slight drop was observed ($p < 0.001$). According to Carfi Pavia et al., 2018, this behaviour could be explained by supposing the reaching of cell confluence at 28 days, followed by differentiation process a physiological detachment due to the lack of available surface. Instead, PLLA-HA 7 %wt scaffolds showed a steady cell growth but slower than PLLA-HA 5 %wt with significant values at 14, 21 ($p < 0.05$) and 28 days ($p < 0.001$). This result confirms that increasing of HA amount is associated with a reduction of cell proliferation.

MC3T3-E1 cells migration and proliferation on PLLA/HA scaffolds after 1, 7, 14, 21, 28 and 49 days of culture are shown in Figure 4.15, where nuclei are visible in blue through DAPI staining. Since the images of two different samples do not reveal significant differences, only pictures of PLLA-HA 7%wt samples are displayed. The images obtained by fluorescence microscopy exhibit already after 21 days the scaffold center colonized, but the cells have not cover the edges yet. Nevertheless, a complete colonization of whole scaffolds surface already after 28 days of incubation. This situation is maintained until 49 days where the cell are so enough to maintain cell-cell contact so the differentiation process can take place and the mineralized matrix can be generated.

In order to understand the MC3T3-E1 slow growth on PLLA-HA 7 %wt scaffold, a marker of osteoblastic differentiation was investigated. Especially, the test was performed only on scaffold containing 7%HA, because as reported in literature (C. Fu et al. 2017; I.

O. Smith et al. 2006), a major effect on the differentiation process is evident in composite with higher filler amount.

Alkaline phosphatase (ALP) activity was monitored until 49 days of culture. The curve in

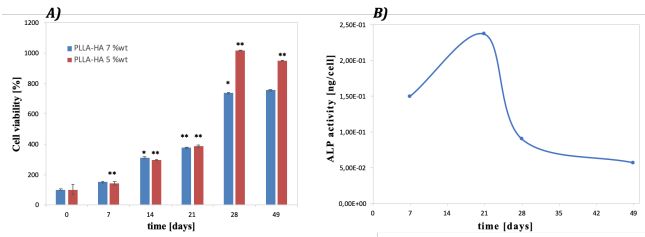


Figure 4.14 B shows ALP activity as nanograms of ALP normalized with number of cells. From 7 to 21 days, the data revealed an increase in ALP activity from 1.5×10^{-1} until 2.37×10^{-1} ng/cell. This results is in agreement with the study of *Peng et al., 2011*, where PLLA/HA nanofibers exhibited an increase of ALP activity between 7 and 10 days of culture. Between 21 and 28 days (

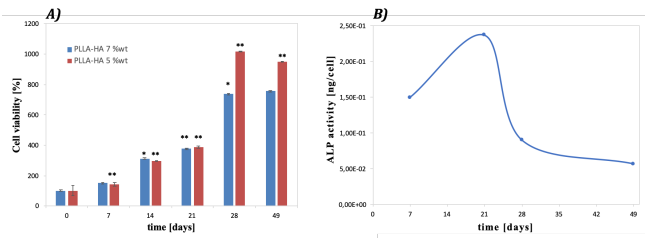


Figure 4.14 B) the amount of ALP decreases until 9×10^{-2} . A similar trend was in ALP activity of 2D osteoblast culture. According to *D. Hong et al. 2010*, the cell regain the

capacity to proliferate after the differentiation in mature osteoblast (21 days). It is confirmed by maximum of cell proliferation value expressed at 28 days. It is consistent with the ability of HA to support osteoblast attachment and promote bone formation within implants. Increasing the HA amount the osteoblast differentiation is favoured but there is also a suppressed growth (Shu et al. 2003). These results demonstrate that PLLA/HA scaffold promote cell growth, even though increasing the HA amount slows down cell grow. Furthermore, HA seems to address the osteoblastic cells toward the maturation when cell confluence is reached. Furthermore, comparing the results obtained in 2D and 3D on scaffold culture, a similar behaviour in term of proliferation and differentiation was obtained. In particular the different steps of MC3T3-E1 differentiation were identified.

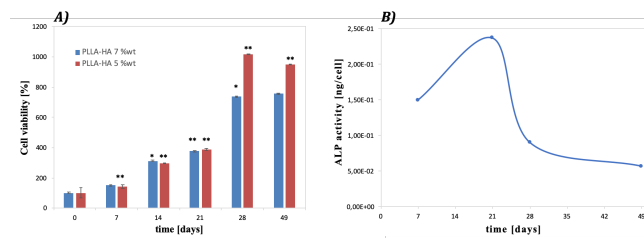


Figure 4.14 (A) Cell viability percentage of MC3T3-E1 cells cultured on scaffold in PLLA-HA 5%wt and 7% wt for up to 49 days; (B) ALP activity of cells on PLLA-HA 7 %wt scaffold at 7, 14, 21 and 27 days. Effect of HA concentrations was investigated. Error bars represent means $\pm$ SD for $n = 3$. Asterisks denote a significant difference compared with the reference, * $P < 0.05$, ** $P < 0.001$.

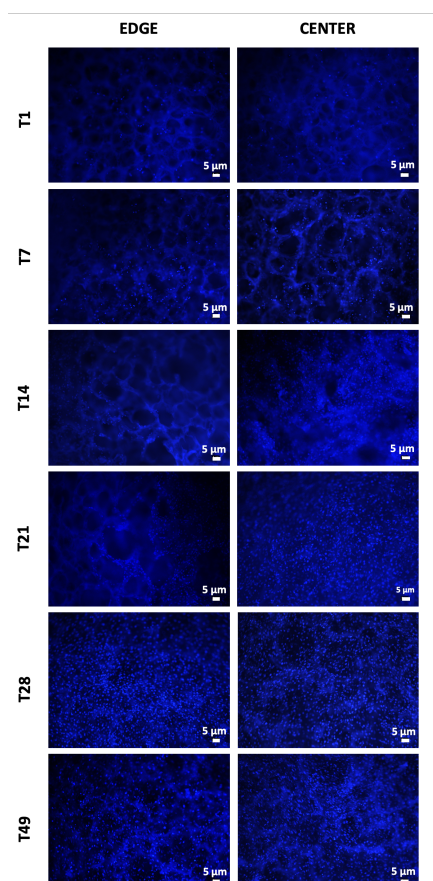


Figure 4.15 MC3T3-E1 cells visualization and quantification by fluorescence microscope on PLLA-HA 7% wt scaffolds after 1, 7, 14, 21, 28 and 49 days of culture. DAPI-stained nucleus in blue. Scale bars 5 μ m.

4.4 HA synthesis and characterization

HA was widely used for application in the bone tissue repair thanks to its biocompatible, osteoconductive properties. Besides, it is easy to synthesize in aqueous solution. Several protocols were followed in literature, although an effect on final morphology of the product was observed changing experimental parameters (Gentile et al. 2015). One of this based on the precipitation method, in which a solution playing the role of phosphate source is added into a solution that is as calcium source. Ammonium or sodium monohydrate phosphate can be used as phosphate source and generally calcium nitrate or acetate can be the source of calcium (Boanini et al., 2010). Moreover, HA synthesis by solution allows to develop properties of HA such as anti- bacterial or mechanical strength, adding in solution oxides, ions and metal or non-metal particles (Bostancioglu et al. 2017; Komur et al. 2017).

The HA was prepared by Wet Chemical Method (Gentile et al. 2015), using calcium nitrate 4-hydrate $[\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}]$, Sigma-Aldrich and diammonium hydrogen phosphate $[(\text{NH}_4)_2\text{HPO}_4]$, Sigma-Aldrich as Ca and P precursors, respectively. A schematic representation of HA synthesis is displayed in Figure 4.16.

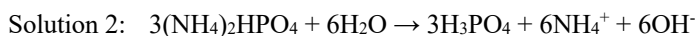


Figure 4.16 Steps of hydroxyapatite preparation via wet chemical method, from Sadat-Shojai et al. 2013

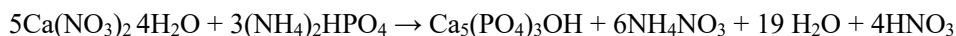
0.03 M aqueous solution of $(\text{NH}_4)_2\text{HPO}_4$ was added drop wise to 0.05 M aqueous solution of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ under stirring (180 rpm) to form a reaction solution. In all experiments the pH of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ solution was adjusted to a value of 10 by NH_4OH 25%v/v solution and the temperature was maintained at 70 ± 5 °C.

All system was aged for 24 h at the same temperature, getting the precipitated HA particles. It was fundamental maintain the experimental parameter constant because a different final products can be get changing the temperature, stirring rape and pH (Eslami et al. 2010).

The reactions that take place into the solution are reported below:



The complete reaction is displayed below:



As the reaction proceeds, the pH of solution has to be adjusted to value of 10 because of formation of nitric acid as waste product, that maintain the pH around 9. Usually, NaOH was used (Nejati et al. 2009) but in this protocol the choosing to adopted a weak base like NH_4OH was made according to (Radha et al. 2017). Although small amounts of NaOH are sufficient to regulate the pH, a lot of sodium tracks are in the final products. Sodium ions could interfere with phase separation process obtaining so scaffolds with irregular

morphology and without interconnected pores. To overcome this problem NH_4OH was used, so sub products can be removed only washing.

At the end, the resulting product (pellet) was centrifuged at a rotation speed of 2500 g for 30 min at room temperature. Then three washing with an aqueous solution of NH_4OH at pH 10 were applied to remove the waste products from HA particles. The so-obtained hydroxyapatite was dried at 70°C in the oven for 24h, mince and directly characterized. The final HA amount was around 30 mg with a percentage yield calculated around 98%

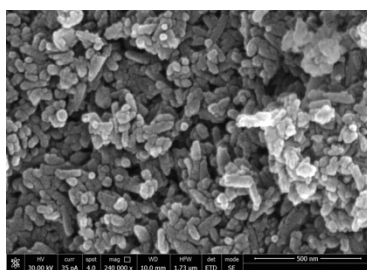


Figure 4.17 SEM micrographs, dried hydroxyapatite at 70°C

SEM micrograph of hydroxyapatite (Figure 4.17) appears without a specific shape, it is hard to define a line of separation amongst particles. More or less, it shows a needle shape, 20 nm wide, around 200 nm length even though the contours of the particles are not well defined.

The EDX spectrum of HA powder is shown in

Figure 4.18. It confirms the presence of calcium (Ca), phosphorus (P) and oxygen (O), without any residues of other elements, unlike what happens when using the NaOH for the correction of the pH as reported by Nejati et al., 2009. Analysing the area under the curve it is possible to calculate the amount of each element and comparing with the theoretical molar percentage of the hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$). Table 4.4 exhibits the results, the matching among the theoretical and the experimental values is satisfactory, and the Ca/P percentage ratio is approximately 1.67.

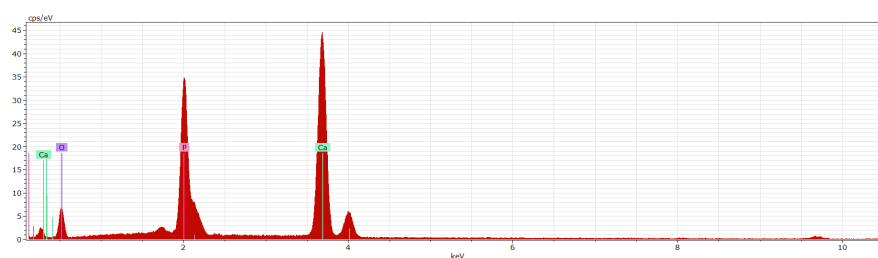


Figure 4.18 EDX spectrum of HA produced via wet chemical method

Table 4.4 Comparison between theoretical and experimental molar ratio of Hydroxyapatite

$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$				Theoretical	Experimental
	Mol	PM(g/mol)	W(g)	% Molar	% Molar
Ca	10	40	400	23.81	46.06

P	6	31	186	18.56	18.52
O	26	16	416	41.52	35.42

The synthesized HA was compared with that bought (CamCeram® III, HA coating powder). XRD analysis of the two samples is shown in

Figure 4.19 A, B.

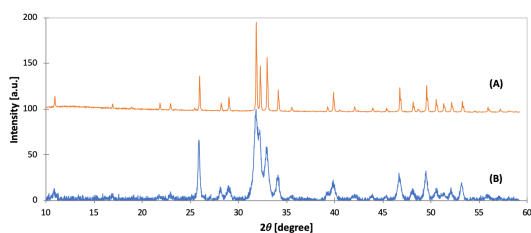


Figure 4.19 : XRD diffraction pattern: A) HA CamCeram® III, B) HA dried via wet chemical method

In both graphs, the three characteristic peaks of HA around 30° and the other peaks around 50° are displayed. The main difference concerns the peaks shape.

In

Figure 4.19A peaks are very sharp and well defined, which means a high level of crystallinity and large crystal. Different is the case of the not calcinated hydroxyapatite

produced by wet chemical method. The pattern displays an increase of the signal at the same angles of the others HA but with a lower intensity. The amorphous part is larger than the other two cases and the background noise a low angle is more accentuated. In any case, the non-calcinated HA presents the same molar ratio and the same configuration of the other.

Before integrating HA in the scaffold, powder obtained was calcinate at 650 °C at the ramp rate of 5.0 °C/min with a dwell time of 2 h in a furnace. The morphology of HA after this step is shown in Figure 4.17. According to Gentile et al. 2015, the calcination temperature of 650 °C was selected to avoid the formation of impurities that could be introduced at higher temperatures. Further no dehydration occurred within the HA molecules during calcination, and the water loss in the samples resulted from the loss of combined water in the HA powders.

4.4.1 Composite PLLA/(insitu) HA scaffolds

The ceramic obtained was used to make scaffolds in order to evaluate interaction with the polymer matrix. Scaffold were produced as reported above with PLLA/HA weight ratio of 95/5. According to previous study (4.3.1 paragraph), the thermal conditions were chosen of 30 °C for 30 min, because allowed to get a structure with pore size appropriate for osteoblasts growing.

Two different HA powder were used: the first one HA powder (CamCeram® III, HA coating powder) obtained via sol-gel (HA_{sg}), the second HA synthesized via wet chemical method (HA_{cm}), described before.

In Figure 4.20 are displayed two scaffold morphology, obtained with the same protocol but with different HA particles.

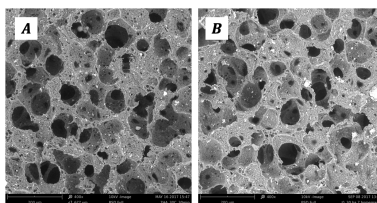


Figure 4.20 Scaffold 5%wt HA 30°C 30min. Two different fillers were used: A) HA_{sg} via sol-gel technique; B) HA_{cm} via wet chemical method

In both case, scaffolds bulk morphology is identical with an average pore size around 100 μm . This demonstrates how HA_{cm} does not interfere with phase separation process and same pores dimensions was obtained.

Although HA integration into PLLA matrix does not change with the filler, a study on effect that this different filler could have on the cell viability needed to be performed.

4.4.2 Cells culture and viability assay

Biocompatible of HA produced by wet chemical methods was investigated, comparing cells viability of MC3T3-E1 on PLLA- HA_{sg} and PLLA- HA_{cm} . As reported above,

scaffolds (5 mm diameter and 1mm thickness) were opportunely sterilized and placed into a 24-well plate. A cell suspension of 9×10^4 cells in 10 μ l was seeded in each scaffold.

Cells proliferation and DAPI staining were carried out to determine the presence and proliferation of the cells on the scaffolds after 1, 7 and 14 days of culture.

4.4.3 Results

Figure 4.21, the MC3T3-E1 cell morphology before the seeding is visualized. As shown the cells are confluent and their shape is regular, even though is not easy to identify a single cell in this condition.

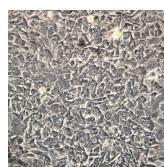
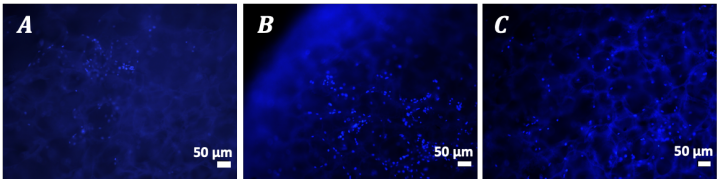


Figure 4.21 MC3T3-E1 cell morphology before seeding

CCK-8 assay was employed to evaluate cell growth levels on PLLA/HA scaffolds with different HA typology. The Figure 4.22 shows the cell viability percentage as a function of the incubation time. The MC3T3-E1 osteoblastic cells cultured on the PLLA, PLLA/HAg, and PLLA/HAc scaffolds for 10 days revealed that osteoblast cells can proliferate on all the scaffolds. An increase in cell viability can be observed with significant differences in terms of viability between the PLLA/HAc and the positive

control (pure PLLA) at 5 days, but also between PLLA/HA_{sg} sample and pure PLLA after 10 days. However, significantly increased cell proliferation was observed on the PLLA/HA_{cm} scaffold compared to the PLLA/HA_{sg} and PLLA scaffolds. The enhanced cell proliferation on the PLLA/HA_{cm} scaffold suggests that the morphology of HA is important in the cell culture, according to a previous work (Haider, Gupta, and Kang 2014).

MC3T3-E1 cells migration and proliferation on three scaffolds after 5 days of culture are



shown in

Figure 4.23, where nuclei are visible through DAPI staining. The images obtained by fluorescence microscopy showed a fair colonization of the surface of all the scaffolds analyzed already after 5 days of incubation. Nevertheless, samples PLLA-HA_{cm} showed a greater colonization capacity compared to the other scaffolds (

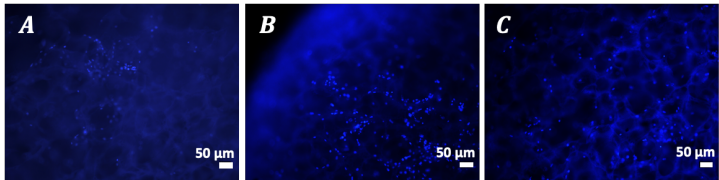


Figure 4.23 B).

These results highlighted that the structural dependent property of HA might affect its potential as bone scaffold.

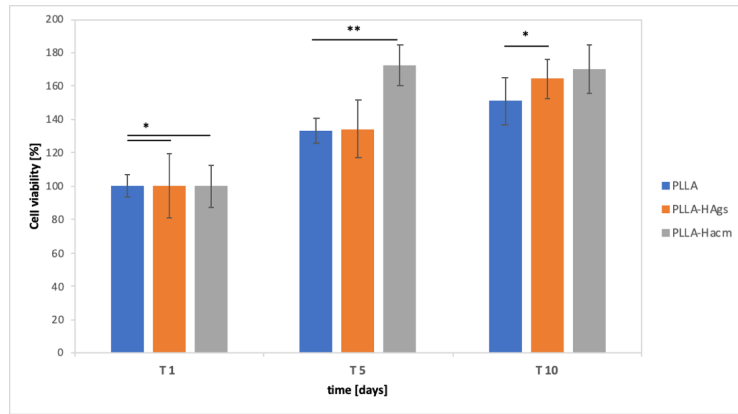


Figure 4.22 Cell viability percentage of MC3T3-E1 cells cultured on scaffold in PLLA/HA ratio 95/5 %wt for up to 10 days. Effect of two types of HA particles was investigated. Pure PLLA scaffolds were used as control. Asterisks denote a significant difference, * $P < 0.05$, ** $P < 0.001$.

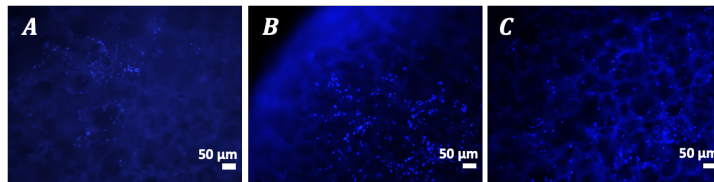


Figure 4.23 MC3T3-E1 cells visualization and quantification by fluorescence microscope on (A) pure PLLA, (B) PLLA/HA_{sg} (C) PLLA/HA_{cm} scaffolds after 5 days of culture. DAPI-stained nucleus in blue. Scale bars 50 μm .

4.5 Outline

Many articles reported as poly (L-lactic acid) (PLLA)-based scaffold was considered a suitable synthetic bone graft substitute, which was largely investigated for bone tissue

engineering applications. Further experimental evidences reported as the nano-hydroxyapatite (HA) holds osteoconductive properties in vitro and in vivo, when it was combined with PLLA (PLLA/HA) the scaffold performance was improved, such as mechanical properties, degradation profiles and permeability, favoring cell viability and activity to facilitate bone repair. For these reasons, PLLA/HA composite matrices have been suggested to be an ideal scaffold in tissue engineering, especially for large bone defect healing.

The approach used for scaffold characterization confirms the μ CT as innovative and not destructive technique to get all set of informations about the material. Unlike traditional methods, the μ CT allows to reduce the analysis time of whole sample. Furthermore, it gives the opportunity to use the same scaffold for biological test being confident that the samples has got exactly the desired characteristics.

Using TIPS as fabrication technique highlighted the advantage to obtaine composite scaffold with a structure with pores highly interconnected just with a simply preparation process. However, these results shown how the scaffold have got the features needed to induce the cell proliferation and osteoblast differentiation.

According to these results, the opportunity to synthetize HA by wet chemical method was considered in order to combined it with the TIPS process. But before to think of that, investigate the cell proliferation into scaffold filled with HA synthetized was necessary.

As discussed above, the results are a good start point to try to implement the fabrication process. In the futures could be think a method to use the HA s synthetized directly after precipitation, without remove the water because it is un-solvent used in the TIPS process. This could be an excellent advantage, because combining this technique with the TIPS, could be reduced a lot the scaffold fabrication time.

Chapter 5

5 Composite scaffold PLLA-BG

Polymeric/ceramic scaffolds are biodegradable, biocompatible and with good mechanical strength making them suitable for biomedical applications. Bioglass® (BG), an inorganic biomaterial characterized by high bioactivity, is able to bind to bone tissue, thanks to the formation *in situ* of a hydroxyapatite layer (fHA) (Macon et al. 2015).

In some studies, polymer/BG composite scaffolds revealed positive additive effects: in particular, was found that BG gets better the integration of polymeric scaffold within the biological environment. Nanofibers composite of collagen-BG have shown a superior osteoblastic activity and good mechanical properties (Sarker et al. 2015). PLGA-BG membrane stimulate the expression of vascular endothelial growth factor (VEGF) increasing the BG concentration than pure polymer (Björkenheim et al. 2017). Furthermore combining BG and polymer improved the mechanical properties of the structure, the amount of water absorption and the bioactivity of the construct in terms of more rapid formation of hydroxyapatite (Conoscenti, Carfi Pavia, et al. 2017; Fabbri et al. 2010; Pitaluga et al. 2018).

In this chapter PLLA-BGs scaffolds produced via TIPS or DIPS are proposed as substitute to repair the bone tissue. The materials were prepared at the University of Palermo whereas biodegradation study, bioactivity test and VEGF expression were carried out in prof. Boccaccini's laboratory at the University of Erlangen-Nuremberg.

5.1 Composite scaffolds produced via TIPS

The possibility to integrate BG45S5 e BG13-93 in PLLA matrix was investigated evaluating the effect of BG composition on the final morphology of scaffold produced via phase separation. In order to obtain highly porous and mechanically stable bioactive devices, a method of integration of BG particles in polymeric scaffold was developed with a *trial and error* procedure.

5.1.1 Bioglass pre-treatment

The bioglass BG45S5 and BG13-93 powders were immersed in a dioxane-water solution with a ratio of 87/13 wt/wt at a concentration of 7 mg/ml for 1 and 7 days. The powders were soaked into solution, sonicated for 10 min and decanted for the established times. Finally, supernatant was removed and BG powders were dried at room temperature.

5.1.2 Scaffold preparation

The scaffolds were fabricated via a TIPS technique, applied to a ternary system. The weight concentrations of PLLA was 6% with a solvent/non solvent (dioxane/water) ratio of 87/13 wt/wt. Different types of bioglass (BG13-93, BG45S5 pre-treated and BG13-93pre-treated) were added to the ternary solution at concentration of 5 %wt. Neat PLLA scaffolds (no filled) were also prepared and characterized and used as control. The protocol to incorporate the BG particles in the polymer solution was similar to the PLLA/HA scaffold (4.1.1 paragraph). The solution with filler was first hot-poured into

different sample holder to evaluate if the different heat transfer can help the filler integration. Sample-holder types were:

(A) aluminium disc-shaped mould (4 cm diameter, 2 mm height);

(B) high density polyethylene (HDPE) cylinder shaped mould with aluminium ring inside (17.6 mm diameter, 35.7 mm height).

Subsequently, they pool immersed in a thermostatic bath and two different thermal histories were followed starting from a temperature of 60 °C:

I) pre-cooling at 30°C for 30min, final quench at -20°C

II) direct quench at -25°C

Final step in an ethylene glycol bath at -25°C blocked any kind of process. After that, the scaffolds were washed in deionized water to remove completely the solvent and dried under vacuum overnight.

5.1.3 Bioglass powders and scaffold characterisation

BG powders

Chemical BGs composition before and after pre-treatment was investigated by energy-dispersive X-ray spectroscopy (EDX) through a punctual analysis. Atomic concentrations of Si, Na, Ca, P, K and Mg were evaluated on the surface of as-received and pre-treated powders for 1 and 7 days in order to individuate a possible changing of ions concentration on BGs surface. Seven fields were analyzed for each sample and the atomic concentration % was reported as mean value $\pm$ SD.

In vitro bioactivity study was conducted on the powder in order to analyze if the pre-treatment influenced the BG bioactivity. Before and after pre-treatment, BG13-93 bioactivity was evaluated with immersion in Simulated Body Fluid (SBF) at 3 and 7 days as previously illustrated in 3.1.8 paragraph. According to Macon et al. 2015, bioactive glass powders were immersed in SBF using a ratio of 75 mg glass to 50 mL SBF in an airtight poly-ethylene container. After the extraction the HA formation was investigated by SEM, FTIR and XRD analysis.

Composite scaffold

Bulk morphologies of porous composite scaffolds prepared at different conditions (BG composition, BG pre-treatment and sample-holder) were examined by SEM (Phenom Pro-X). Morphological changes of the microstructure were investigated.

Mechanical tests were carried out on five samples (n=5) to compare the scaffolds loaded with 13-93BG w/wo pre-treatment. Compression test was performed using cylindrical samples (diameter 13 mm, thickness 3 mm) as described in 3.1.5 paragraph.

5.1.4 Results

As known the BG releases ions in aqueous solution already after few hours from immersion (Qazi et al. 2017). In particular calcium ion release in BG13-93 was found to be swifter and in greater concentrations compared to BG45S5, whereas it showed a faster release rate of Na and P ions compared to BG1393. No difference in Si ion release concentrations or rates were observed between the two BG types. The exchange between

Na^+ ions from BG bulk and H^+ from the solution leads to overall increase in pH (Abdollahi et al. 2013; Cerruti et al. 2005). This phenomenon interferes with the TIPS process, in fact the scaffold produced are and brittle and with poor mechanical properties (Conoscenti, Carfi Pavia, et al. 2017). It was essential finding a solution to overcome this problem, so according to Abdollahi et al. a pre-treatment of BG was performed.

Bioglass pre-treatment

In Figure 5.1 A, surface morphology of bioglass particles was displayed. Figure 5.1B shows the content of the cations on the surfaces of each glass before and after pre-treatment (1, 7 days). O was removed from the calculations.

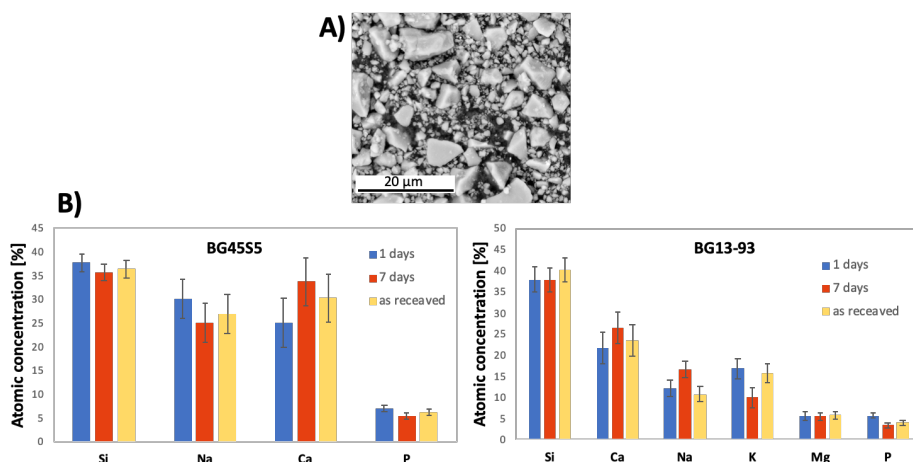


Figure 5.1 A) SEM image of BG13-93; B) Atomic % concentration measured on the BG surface as received and after pre-treatment in dioxane-water 87/13 %wt for 1,7 days. O was removed from the calculations.

No significant difference on the atomic concentration % was highlighted between powders pre-treated versus non pre-treated.

Scaffold morphology

The morphologies of composite scaffolds with the two types of pre-treated BG are shown in Figure 5.2. For PLLA/BG13-93 samples the presence of the particles does not seem to influence morphology of the scaffold and consequently the phase separation process.

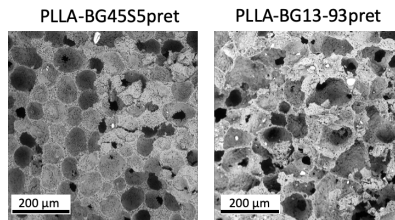


Figure 5.2 Comparison between the morphologies of scaffold PLLA-BG45S5pre-treated and PLLA-BG1393pre-treated produced via TIPS 30°C 30min in aluminium holder sample

A different behavior was detected for the BG45S5, where a lot of micropores can be observed at the level of the scaffold walls. It follows that the presence of BG altered the nucleation rate during the nucleation and growth process, probably due to a higher reactivity of BG45S5 with respect to BG13-93. So, the pre-treatment helps only the integration of BG13-93 because it exhibits a lower reactivity.

In Figure 5.3 the PLLA-BG13-93 scaffold morphologies obtained with different sample-holders, bioglass pre-treatment and thermal histories are displayed.

Scaffolds with not-treated bioglass produced following the thermal path (I) into the aluminum sample-holder and HDPE sample-holder exhibit an average pore size of $\approx 60 \mu\text{m}$ vs $\approx 140 \mu\text{m}$ respectively. Unfortunately, their structure was very brittle. Also, the pre-treatment influences strongly the final morphology. As a matter of fact, pre-treated BG13-93 samples produced into the aluminum sample holder following the (I) path, present a pore size of $50 \pm 1.2 \mu\text{m}$ whereas a pore size of $120 \pm 20 \mu\text{m}$ can be obtained when not treated BG was used.

No significant differences were observed when following the thermal path (II). In all cases a pore size of $\approx 10\text{ }\mu\text{m}$ was obtained. This result is probably due to the fact that the sudden quench leads the solution directly to the spinodal region where the effects given by the different sample-holder and BGs are removed.

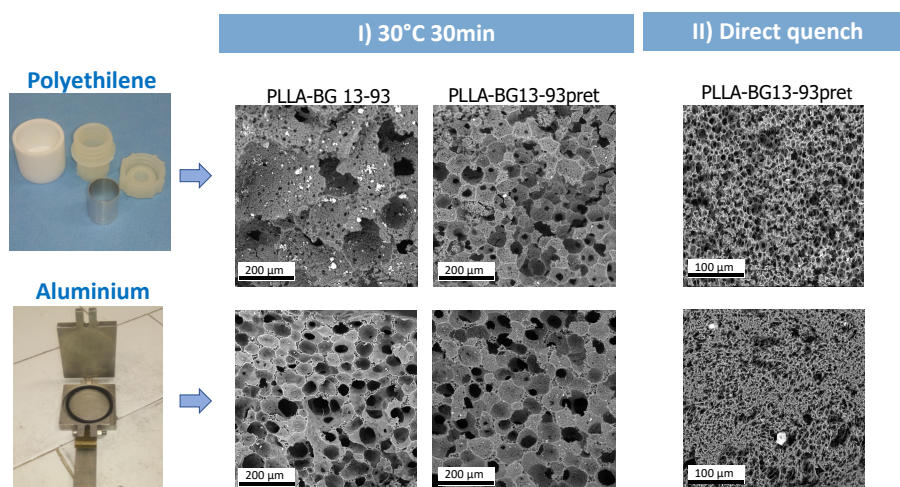


Figure 5.3 SEM micrographs of PLLA/BG scaffold obtained via TIPS changing to sample-holder, demixing temperature and time.

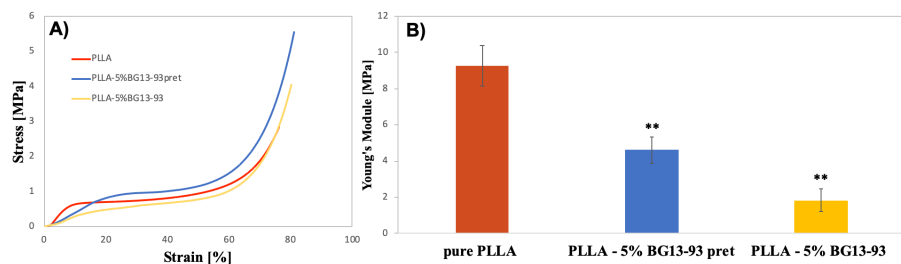


Figure 5.4 A) Stress-strain curves of PLLA, PLLA/5%BGl3-93 and PLLA/5%BGl3-93pret; B) Compressive Young's modulus, ** $p < 0.01$

Compressive strength tests were carried out on pre-treated BG13-93 and not-treated BG13-93 scaffolds and compared to pure PLLA scaffold. A typical compressive stress–strain curve is shown in Figure 5.4 A and the calculated compressive moduli are shown in Figure 5.4 B.

The PLLA scaffold had a compressive modulus of 9.27 ± 1.1 MPa. The addition of BG into the scaffold reduced the mechanical properties. It is easy to observe that the modulus significantly decreased (more than double) when adding a BG amount of 5%. An higher Young's module was obtained for samples with pre-treated BG13-93 with respect to samples filled with not treated BG. Mechanical properties of the porous system depend on several parameters such as: porosity, pore size, amount of filler, thermal history and/or other parameters concerning the foaming process (Rogina et al. 2016). It makes a meaningful comparison of literature data rather difficult. It is reasonable to speculate that,

the poor interfacial adhesion between PLLA and BG particles may have a weakening effect.

Bioactivity test

Figure 5.5 are compared the surfaces of BG13-93 pre-treated before and after 7 days of immersion in SBF. Only SEM images of the BG13-93 pre-treated are reported, being representative of other samples. After 7 days, the typical cauliflowers-like shape of HCA was individuated (Figure 5.5 B) (Roohani-esfahani et al. 2011).

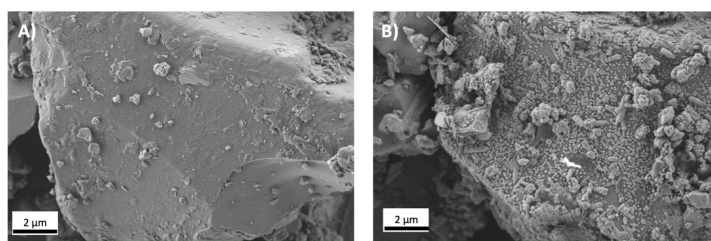


Figure 5.5 SEM micrographs of BG13-93 pre-treated before (A) and after 7 days in SBF (B)

Therefore, to confirm the nature of the newly crystalline phase formed at the surface the different glasses, X-ray diffraction was carried out as shown in

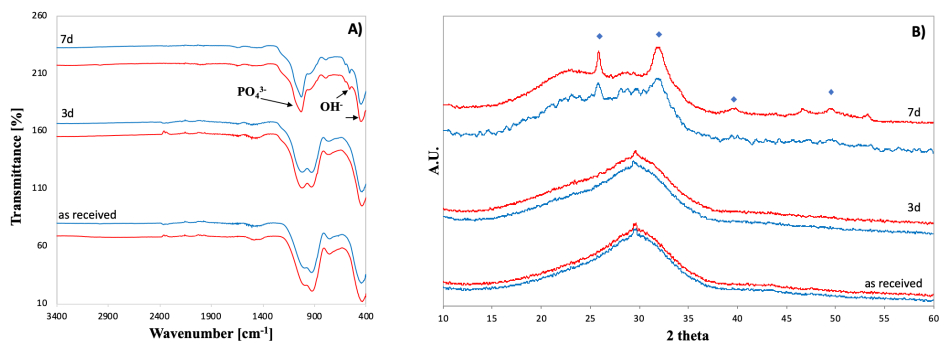


Figure 5.6 B. XRD results confirmed abundant HCA formation on the surface of the glass at 7 days. Sharp peaks centered at $2\theta = 29^\circ$, 32° and 46° , highlighted by blue points, underline the formation of HCA-like layer, confirming the SEM observation. The bioactivity of BG reported here was in agreement with literature (Wetzel and Brauer 2019).

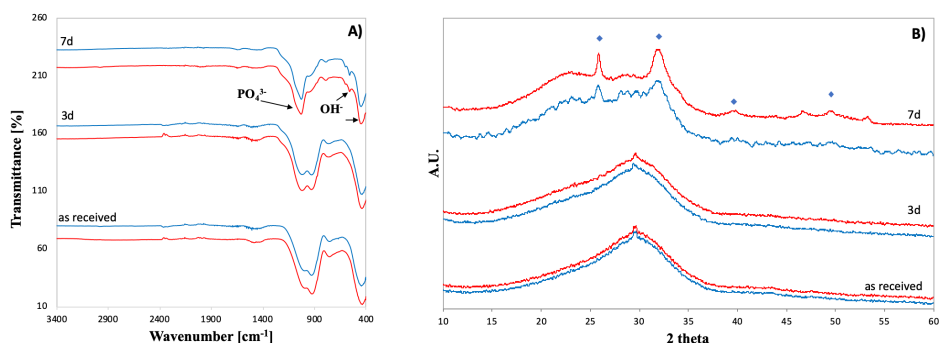


Figure 5.6 FTIR spectra (A) and XRD pattern (B) of BG13-93 powders as received, 3 days and 7 days after immersion in SBF. In red powder pre-treated in dioxane/water solution, in blue non-pre-treated powders

FTIR data were in good accordance with XRD results. The

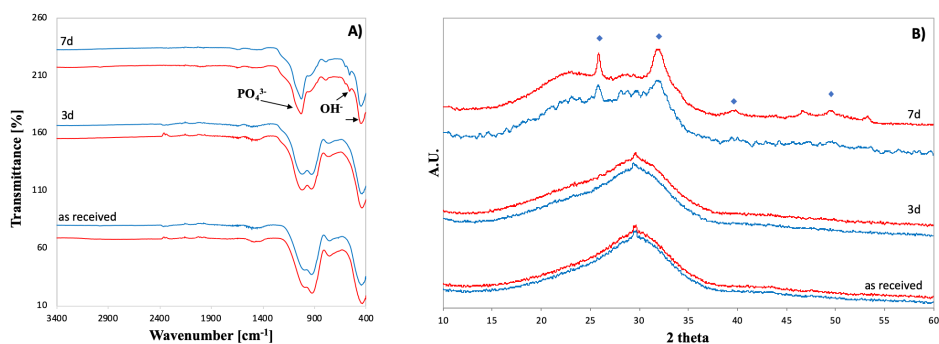


Figure 5.6 A shows the FT-IR spectra of BG13-93 powders. Characteristics adsorption bands of HA were exhibited at 622 cm^{-1} due to hydrogen-bonded OH^- ions, a band at

1040 cm^{-1} attributed to $\nu_3 \text{ PO}_4$ and the bands at 603 cm^{-1} and 561 cm^{-1} to $\nu_4 \text{ PO}_4$ (Eslami et al. 2010).

However, given that FTIR and XRD spectra do not highlight differences before and after pre-treatment, it can be concluded that pre-treatment does not influence the BG bioactivity. As matter of fact HCA is visible with its characteristic shape after the soak in SBF (Zhou et al. 2007) in both cases.

5.2 Composite membrane produced via DIPS

Polymer membranes have been widely used as guide for bone regeneration (GBR) especially in the periodontal field. At the beginning the use of membranes was specifically devoted to the generation of new bone around implants (J. Wang et al. 2016). On the other hand, the use of BG in membrane has strongly increased also for its angiogenic potential (Saghiri et al. 2016), as it has been shown that a change in BG chemical composition may turn onto a modification in its bioactivity. For instance, the incorporation of various ions, like boron (B), could be of paramount importance for osteointegration (Xia et al. 2019) or might improve cells metabolic activity (Hoppe, Güldal, and Boccaccini 2011). The angiogenesis plays a central role in bone regenerations by providing the functional connection between the grafting material and surrounding host tissues. It is a process necessary to achieve a rapid revascularization, allowing the transport and exchange of oxygen, nutrients, growth factors and cells involved in tissue repair and regeneration.

With this respect preparation and characterization of composite membranes made by a poly-lactic-acid (PLLA) matrix and a bioactive glass filler as possible bone substitutes will be described in the following.

5.2.1 Membrane preparation

Composite membranes were prepared via Diffusion Induced Phase Separation (DIPS), a technique currently used to produce porous membranes and briefly illustrated below (Figure 5.7). Main steps can be summarized in dip-coating, immersion into two coagulation baths and desiccation. First of all, binary solution with 8%wt of PLLA was prepared by hot dissolving the polymer in 1,4-dioxane. Subsequently, filler was suspended into the solution with a weight ratio between polymer-bioactive glass of 90/10. BG powders were added to a homogeneous solution at a temperature of 65°C. Three distinct solutions were prepared and sonicated at 35 kHz for 1h. This step was fundamental to get rid of particles aggregates and guarantee a significant BG powder dispersion with the suspension. Afterwards, the polymer-BG suspension was placed into a water bath and kept at 30 °C (Figure 5.7 A). A stainless-steel slice AISI 304 (15 x 70 mm, thickness 3 mm) was immersed into polymer-BG solution and pulled-out at a constant velocity of 10 cm/min. The slice surface was thus coated with a PLLA-BG layer with a thickness depending on the pull out velocity, temperature and concentration, as reported in literature (Carfi Pavia et al. 2012). Afterwards, the membrane was soaked in

two coagulation baths to allow the diffusion of solvents from and to the casted solution, inducing liquid-liquid phase separation and/or polymer crystallization. According to previous works, the membranes were kept in a first bath (dioxane/water in weight ratio 87/13) for 10 min and in a second bath (deionized water) for 5 min (Montesanto et al. 2015) (Figure 5.7 B). Lastly, the as-obtained membranes were desiccated at room temperature (Figure 5.7 C). The final thickness of these membranes was $180 \pm 20 \mu\text{m}$.

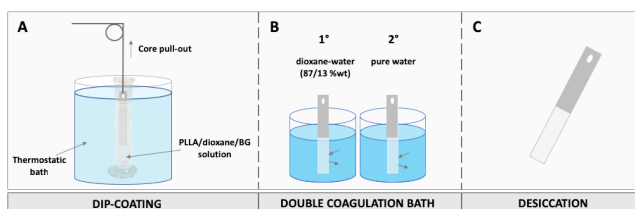


Figure 5.7 Schematic representation of Diffusion Induced Phase Separation (DIPS) process

5.2.2 Membrane characterisation

Membrane characterization were carried out by technique illustrated in 3 chapter. Micro-surface morphologies of all membranes were assessed by SEM analysis. The two sides of membrane were identified as surface into contact with metal sheet (inner surface) or with air (external surface), respectively.

Measurements of thermogravimetric analyses were carried out by using a sample with initial weight about 10 mg.

Sample morphology was also investigated by micro-computed tomography via high-resolution μ -CT scanner. The images resolution was 3280 x 4904 with a pixel size of 0.85 μm .

5.2.3 In vitro bioactivity study

In vitro bioactivity of composite membrane was examined adopting the standard procedure described in 3.1.8 paragraph. All membrane typologies (dimensions 14 x 5 mm) were immersed in SBF solution into well-sealed plastic tubes to minimize the pH variation and reduce the microorganism contamination. Samples were soaked in a volume of SBF previously calculated, based on a ratio between apparent surface area of specimen and solution volume of 10 (Kokubo and Takadama 2006). Afterwards, the specimens were incubated for 3, 7, 14, 21 and 28 days.

5.2.4 In vitro study of angiogenic potential

5.2.4.1 Cell culture and samples preparation

Mouse bone marrow cells line (ST2) were cultured in cell culture medium (CCM) comprising of RPMI 1640 (Gibco, Germany), supplemented with 10 vol% fetal bovine serum (FBS, Sigma- Aldrich, Germany) and 1 vol% penicillin/streptomycin (PS, Gibco, Germany). The cells were cultured in cell culture flasks T-75 (Nunc, Denmark) inside a static incubator at 37°C with 5% CO₂. The medium was replaced every two days until reaching a confluence of 70-80%; cells were split in new flasks to obtain the cell number

needed for the experiments. Then, the cells were detached via Trypsin/EDTA treatment and suspended in complete medium for *in vitro* experiments. Cells were counted by using the hemocytometer corresponding to the Neubauer chamber, therefore cell suspension was mixed with a solution of trypan blue in 1:1 ratio to count only living cells.

The so-produced membranes were opportunely decontaminated in two steps by adapting the procedure used by Roohani-Esfahani et al. (Roohani-esfahani et al. 2011)(Wilda and Å 2006), in order to minimise the risk of contamination during cell culture. First of all, composite materials were exposed to UV light for 1 hour and they were rotated every 30 min to disinfect both membrane surfaces. Afterwards, the membranes were impregnated with 70% ethanol for 1h and rinsed three times with phosphate-buffered saline (PBS) for 15 min to remove any solvent residue. In parallel, all BG powders were sterilized at 160°C for 2 h in a furnace (Balasubramanian et al. 2017).

5.2.4.2 Cell seeding

Biological investigation was carried out by indirect cell culture, following protocols previously developed by Balasubramanian et al. 2017 (Figure 5.8). In order to obtain the medium with dissolution products, all specimens (powders and composites) were preconditioned with complete medium for 24 h in an incubator. The cell concentration was of 100,000 cells/ml. 1ml of ST2 cell suspension was seeded on 24-well plates and incubated for 24 h at 37°C in 5% CO₂ atmosphere. Simultaneously, 0.1 g of each BG powders was added to 10 ml of complete medium to obtain suspensions of 1 wt/vol%. Composite membranes were shattered and supplemented medium was added to obtain

suspensions with 10 wt/vol%. Both suspensions were incubated for 24 h at conditions similar to those undergone by the cells. After 24 h, solutions with 0.1%, 0.01 wt/vol% concentrations (for powders) and 1%, 0.1%, 0.01 wt/vol% (for composite) were obtained by dilution of stock solutions with CCM. At this point, the medium was removed from the attached ST2 cell and replaced with the solutions at different concentrations containing the dissolution products. In order to evaluate the effect of dissolution products, the cells were incubated for 48 h. Furthermore, cells cultured in CCM without dissolution products were used as positive control, while cells with cultured in CMM with 10 vol% dimethyl sulfoxide (DMSO, Sigma-Aldrich) as negative control.

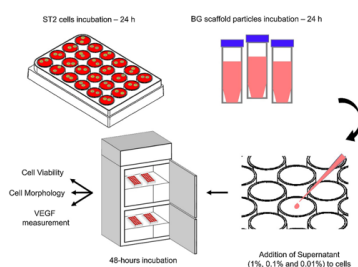


Figure 5.8 Schematic representation of the experimental set-up for the cell biology study, from Balasubramanian et al. 2017

5.2.4.3 Cell viability and morphology

Cell viability was analysed using a WST-8 kit after 48 hours of culture with dissolution products. The test was performed in triplicate for each condition (n=3). Hematoxylin/Eosin staining allowed investigating the cells morphology after 48 hours of

culture with solutions containing different concentration of BG dissolution products, according to protocol reported in literature (Ureña et al. 2018).

5.2.4.4 VEGF expression (ELISA test)

The quantitative measurement of mouse VEGF in cell culture supernatants (collected before viability test) was performed by using a commercial RayBio® Mouse VEGF ELISA kit, an *in vitro* enzyme-linked immunosorbent assay. According to the manufacturer's instructions, 100 µl of standards and samples were pipetted into 96-well plate coated with anti-Mouse VEGF and incubated for 2.5 hour at room temperature with gentle shaking. In this way, VEGF present in the sample is bound to the wells by an immobilized antibody. The solution was discharged and the wells were washed 4 times with wash buffer. Subsequently, 100 µl of biotinylated antibody was added and incubated for 1 hour at room temperature. After washing away unbound biotinylated antibody, 100 µl of HRP-conjugated streptavidin was pipetted to the wells and incubated for 45 min at room temperature. The wells were again washed and 100 µl of TMB substrate solution was added, followed by incubation for 30 min at room temperature in the dark with gentle shaking. In this step, the colour of the solution changes in proportion to the amount of VEGF secreted by ST2 cells cultured in presence of dissolution products of BG particles or composites of BG/PLLA. Finally, 50 µl of stop solution was added in each well, leading to the turning from blue to yellow and the absorbance was immediately measured at 450 nm by a microplate reader. Absorbance values of samples and standards were translated into pg/ml of VEGF release. Final results were expressed as relative values %.

5.2.5 Results

5.2.5.1 Characterization of samples: morphology and bioactive glass distribution

SEM micrographs of PLLA/BG 13-93 membrane are shown below (Figure 5.9). Due to double coagulation baths, a homogeneous membrane morphology was obtained on both sides with pores of $5 \pm 2.5 \mu\text{m}$ and a homogenous filler distribution, which is significantly appreciable in the inner surface. The amount of filler effectively loaded in the polymer solution and integrated in the composites was evaluated by TGA measurements. Thermogravimetric analysis (% mass loss vs. temperature) of films prepared by DIPS (Figure 5.10) showed a weight loss of $\sim 90\%$. Trend of PLLA-BG13-93 is reported as an example, since TGA curves of others films showed similar tendency, regardless of the type of BG. This result is in line with with Deliormanlı et al., 2012, who indicated that the composition of the bioactive glass had insignificant effect on the thermal debinding kinetics. The region of largest weight loss ($\sim 290^\circ\text{C}$ to $\sim 370^\circ\text{C}$) corresponds to the removal of PLLA since that degradation took place at $\sim 300^\circ\text{C}$ (Chieng et al. 2014), while no weight loss is associated to BG powder. Thereby, the value of BGs estimated in each sample was of $9 \pm 0.5 \text{ \%wt}$. This result highlights that during the membrane preparation the loss of filler is quite small, as the amount of BG is comparable with the nominal quantity.

The BG distribution may also be displayed by means of micro-CT 3D reconstruction (Figure 5.11 A, B). The filler well distributed within the polymer matrix is highlighted in red. Also, a film thickness of around $180\mu\text{m}$ was measured (Figure 5.11 C).

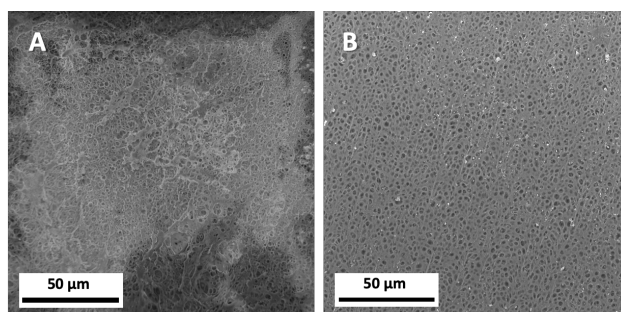


Figure 5.9 SEM micrographs of PLLA/BG 13-93 membrane: (A) external surface, (B) inner surface.

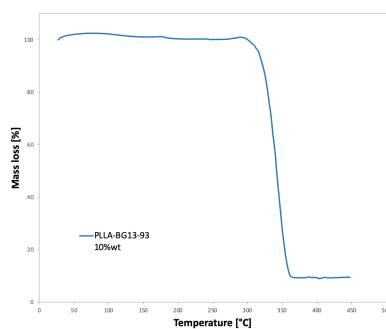


Figure 5.10 TGA analysis: Mass loss (%) vs Temperature (°C) of PLLA/BG 13-93 membrane.

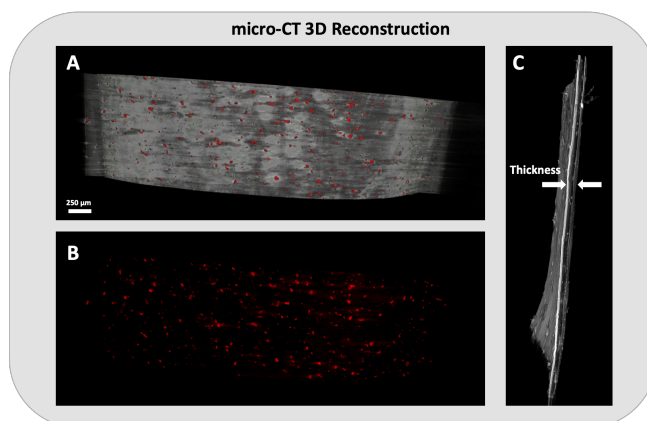


Figure 5.11 Three dimensional reconstruction of membrane PLLA/BG-13-93 with 10% filler by CTvox: (A) PLLA sheet (grey) BG particles (red), (B) distribution of BG particles, (C) membrane thickness, scale bar 250μm.

5.2.5.2 In vitro bioactivity study of composite membrane

FTIR spectra of all samples and XRD patterns are reported in Figure 5.12 before and after 14 days of immersion in SBF. For the sake of brevity, only the results after 14 days in SBF are reported, because HCA formation on the composite membrane is already visible. The FTIR spectroscopic analysis of pure PLLA and PLLA/BGs membranes were carried out to identify the functional groups as well as to analyze the possible formation of hydroxyapatite on the surface after immersion in SBF. Characteristic absorption bands for PLLA appear in the region $3000\text{--}2875\text{ cm}^{-1}$ due to C-H stretching of CH_3 . The O-H group of carboxylic acids could have also contributed to this band. From C=O stretching vibration of carbonyl the pick at $1750\text{--}1745\text{ cm}^{-1}$ is originated. The absorption bands at

1450–1375 cm^{-1} are related to the symmetric deformation of C-H stretching in the CH_3 , the peaks at 1100–950 cm^{-1} are attributed to C-O-C stretching while the peaks corresponding to C-C single bond appears at 920 and 871 cm^{-1} (Sanyanga et al. 2016; Herrera-Kao et al. 2018). Stack plot of FTIR spectra displayed in Figure 5.12 does not reveal any new peak for composite samples, compared to pure PLLA. According to Chieng et al. 2014, Haafiz et al. 2013, the addition of low concentrations of BG causes only physical interactions with PLLA. Besides, the peaks of PLLA largely overcome the peaks of BG, which are not easily distinguishable in the composites.

On the other hand, after 14 days in SBF, the pure PLLA curve shows the previously mentioned bands, while the composite membranes reveal diverse new bands. Bands at 3635 cm^{-1} have been assigned respectively to OH anti symmetric and OH symmetric stretching (Ryszkowska et al. 2010). The bands of PO_4^{3-} at 564, 605, 1034, and 1098 cm^{-1} while the absorption bands of CO_3^{2-} at 1448 cm^{-1} suggest the existence of HCA, being specific of the FTIR spectrum of HCA layer (L. Ji et al. 2015; Cox et al. 2015; Azis et al. 2015). Double peaks appear at 1460 and 1420 cm^{-1} , indicating that B-type HCA was formed because CO_3^{2-} replaced sectional PO_4^{3-} . Also, a displacement of signal have been observed from 1746 cm^{-1} to 1755 cm^{-1} that could be attributed to displacement of the carbonyl signal of the PLLA, which is attributed to the interaction of the hydrogen bond of the carbonyl group and the surface P-OH groups of HA (Zhou et al. 2007).

The XRD spectrum of as prepared pure PLLA indicates an intense diffraction peak at $2\theta = 16.6^\circ$ corresponding to the main peak of pure PLLA (J. Huang et al. 2013). By adding of BGs in the polymeric membrane no influence on the crystallinity of PLLA was

detected. In fact, all curves show the same trend. After 14 days in SBF, distinct effects for pure and composite samples are highlighted. Substantially, XRD spectra of neat PLLA before and after soaking in SBF for 14 and 28 days exhibited almost no change. This behavior is due to the low bioactivity of neat polymer. On the other hand, XRD patterns of PLLA/BG membranes revealed peaks between 25° - 40° and 50° - 60° , notably at $2\theta = 28.2^\circ$, 34.8° and 55° . These peaks are assigned to the diffraction of the (210), (202) and (322) planes of HA crystalline phase, according to JCPDS 09-0432. This result confirmed that the formation of HCA on the PLLA/BGs surface should be attributed to the significant bioactivity of the BG dispersed in the PLLA matrix.

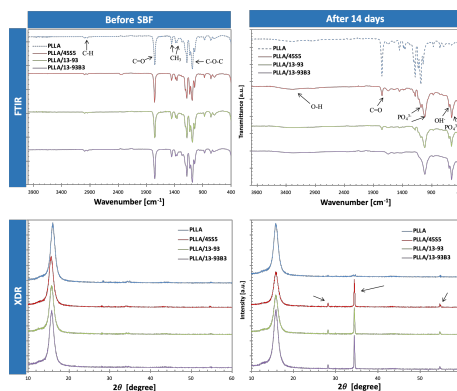


Figure 5.12 FTIR and XRD spectra of pure PLLA and PLLA/BGs membrane: (left) before and (right) after 14 days of soaking in SBF. The peaks indicated in this figure are discussed in detail in the text.

Additionally, SEM images illustrate the evolution of the microstructure of samples after 14 days in SBF (Figure 5.13). The morphology of pure PLLA is not displayed since no change is detectable, as confirmed by the FTIR and XRD analyses discussed above. After 14 days, composites surface is covered by HCA in same points, in fact it appears wrinkled and not as smooth as before.

Apatite-like structures like cauliflower ($\sim 3.5 \mu\text{m}$ size) is detectable on the surface of PLLA/BG45S5 (Figure 5.13 D) (Roohani-esfahani et al. 2011). In the case of 1393BG composite scaffolds, the original pore structure remains still evident. Moreover, the presence of inorganic compounds in various spots on the whole membrane surface (Figure 5.13 E-F) and not only around the BGs particles is a rather remarkable outcome. In accordance with the present results, previous studies ((Ryszkowska et al. 2010))(L. Ji et al. 2015)) have demonstrated that bioactive glass/polymer composites present outstanding apatite-forming ability after soaking in SBF, due to the presence of BG. This effect increases with BG amount incorporated in the polymeric matrix.

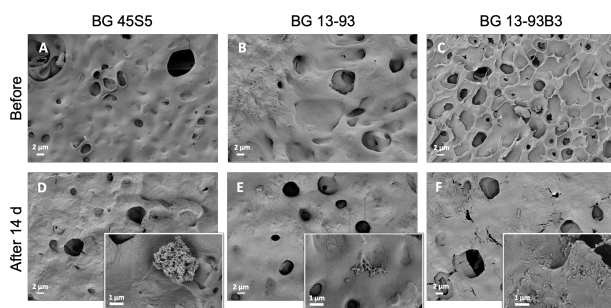


Figure 5.13 SEM micrographs of the surface of composite scaffolds after soaking in SBF for 14 days at different magnifications. (A) PLLA/BG45S5, (B) PLLA/BG 13-93, (C) PLLA/BG 13-93B3

5.2.5.3 In vitro study of angiogenic potential

Cell viability

Figure 5.14 A, B show the cell viability of ST2 cells in culture with dissolution products of BG powders only and composite membranes with different BG concentrations, respectively. The behavior of cells cultured only with CCM was taken as reference and it was normalized to 100%. This value was used as positive control and significant differences between samples were established in comparison to it.

Bioactive glass powders

A quantitative assessment highlights an increase in cell viability with decreasing BG concentration after 48 h of culture; this behavior was observed for both 45S5 and 1393

glasses but not for borate 13-93-B3 bioactive glass. For this latter, the highest viability is observed at 0.1% concentration ($P<0.001$) and nearly the same (lower) value at 1% and 0.01% concentrations ($P<0.001$), but always significantly higher than reference. This effect can be linked to the initial release of B ions in the 13-93B3 during the 24 h of immersion with a maximal concentration within four days, as reported by *P.Balasubramanian et al. 2016* and *L. (Xia et al. 2019)*.

Furthermore, no effect on the cell viability was displayed for 45S5 at higher concentration (1%), probably owing to the higher pH value of the cell culture medium ((Ciraldo et al. 2018)) according to a prior study on hMSC cells ((Qazi et al. 2017)). All the other BGs show a major increase of cell viability than reference, but the greater cell viability is evident for 45S5 and 13-93 at the lowest concentration ($P<0.001$) (Figure 5.14 A).

Composite membrane

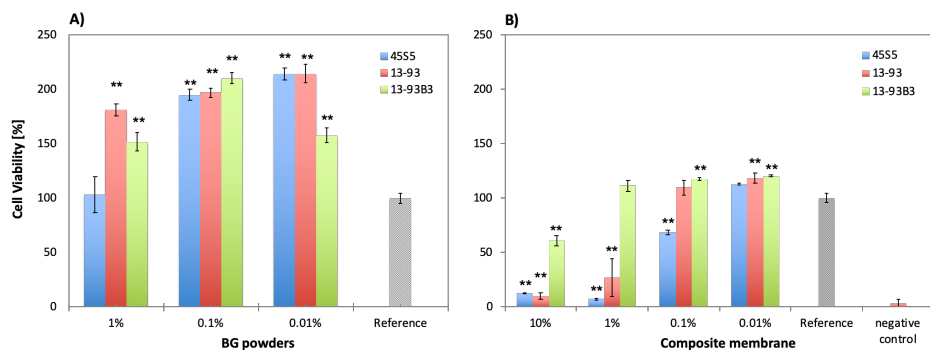
Also, in the case of cell culture in solution with IDPs of membranes, an increase of cell viability is achieved with decreasing BG concentration. The highest rate was registered at 0.01% concentration for all samples, even slightly higher than the positive control. Especially, stimulation of ST2 cells with IDPs of PLLA/45S5 and PLLA/13-93 did not contribute significantly to an increase in cell proliferation, rather the cytotoxic effect is shown at concentration of 10% and 1% ($P<0.001$).

Indeed, the cell viability is low (above the 4%) for the negative control, which is in presence of DMSO as described above.

PLLA/13-93 membrane shows already an improvement of cell proliferation at 0.1% compared to the 45S5, which shows cell viability still lower than the positive control.

According to literature ((Silver, Deas, and Ercin 2001)), a possible explanation for this might be attributable to the ions released during immersion of BG in CCM (Cerruti, Greenspan, and Powers 2005), (Abdollahi et al. 2013)). Composites containing borate BG (13-93B3) shows a greater cell viability at all concentrations compared to others composite, contrary to what reported in literature in the case of only borate BG powder ((Q. Fu et al. 2010)) since the degradation of the borate BG and therefore the release of ions is retarded inside the membrane.

Finally, comparing the global values in both cases, low concentrations of BG powders showed an increased cell viability compared to those of the composites (Figure 5.14).



*Figure 5.14 Relative viability of ST2 cells cultured with dissolution products of: a) BG powders; b) PLLA/BGs 10% wt membranes at different dilutions. Asterisks denote a significant difference compared with the reference, ** $P < 0.001$.*

Cell morphology

Light microscopy images of H&E-stained ST2 cells are displayed in Figure 5.15. Only pictures of cells cultured with 1% and 0.1% dilution of all three composition of BG powders and three typologies of composite membrane are reported, since no significant differences were visualized for the other dilutions. From the pictures, a good cell adhesion to the well plate and the phenotypical cell morphology can be observed. The non-cytotoxic effect of the BG powders was confirmed, since cell morphology not change with concentration and BG typology. In each samples, the surface appears completely covered by ST-2, as reported in a previous work ((Balasubramanian et al. 2017)). While, in presence of IPDs of composite membranes, the cells did not seem homogeneously attached on the surface until confluence. A relatively poor cell proliferation and adhesion could be qualitatively noted for 45S5 and 13-93 composite membranes at 1% concentration. While membrane with 13-93B3 shows a higher cell density just like membrane at 0.1% concentration. However, these qualitative considerations based on visual observation partly confirm the quantitative results of WST assay, especially for composite membranes.

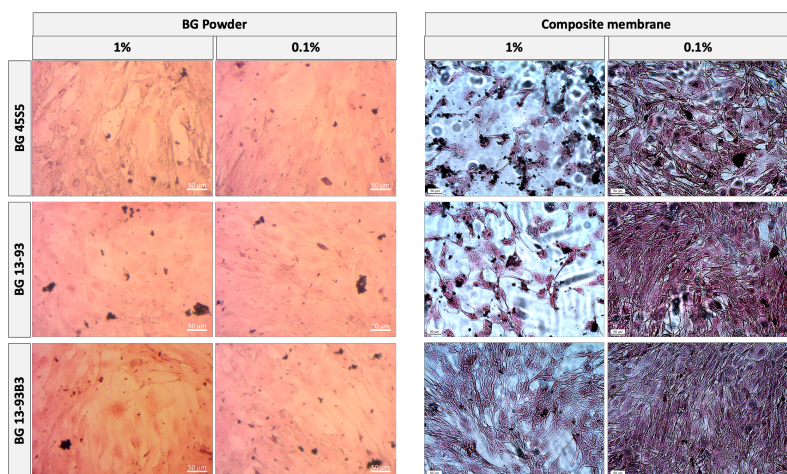


Figure 5.15 Comparison between light microscopy images of H&E-stained ST2 cells cultured with 1% and 0.1% dilution of different BG powders and composites membranes.

VEGF expression

The angiogenic potential of materials was evaluated by measuring the secretion of the growth factor VEGF. Especially, angiogenic potential of different BG powders and composite membrane PLLA/BG 10% wt was compared in order to investigate, how the 8% wt of polymer could influence the BG effect on cell culture. VEGF secreted by the ST2 cells in the presence of IDPs of BG particles or PLLA/BG membranes are displayed in Figure 5.16 A-B, respectively. In both cases, the VEGF release increased with the concentration in borate bioactive glass, but the values achieved by composite membranes are lower in respect to BG powders.

Bioactive glass powders

At concentration of 0.01%, all membranes typology shows a percentage of VEGF release comparable with the control. Borate bioactive glass presents a different behaviour than 45S5 powder, showing an increment of VEGF secretion with BG concentration. In particular, only significant differences could be found for 1% and 0.1% 13-93B3.

Composite membrane

Once again, exposure to ionic release products from all BGs did enhance ST2 metabolic activity compared to the control, even though the presence of PLLA mitigates the effect of BG ions.

In fact, the highest value of VEGF secretion is displayed for PLLA/13-93B3 membrane at 10% of concentration. This result confirms that borate BG stimulates the expression of vascular endothelial growth factor in cells but at high concentration. PLLA/1393-B3 at 0.1% stimulated cell viability and therefore the cells release a low percentage of VEGF.

As reported previously in literature, biological responsiveness of composites is typically lower than pure powder in the indirect test (Bellucci et al. 2019).

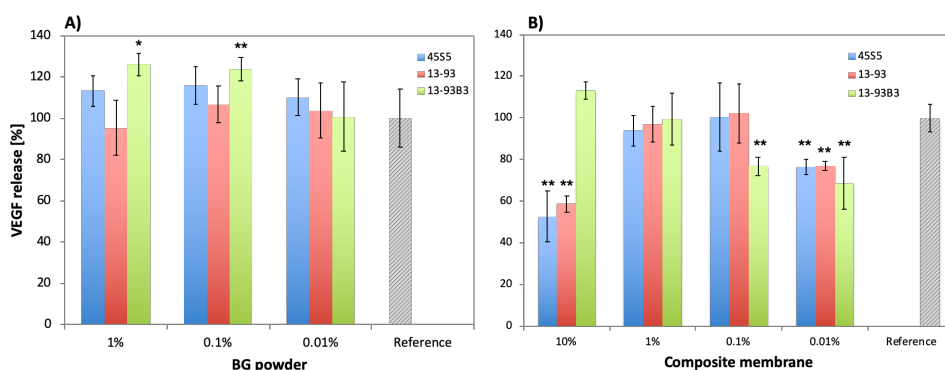


Figure 5.16 VEGF release from ST2 cells treated with different dilutions of IDPs of: A) BG powders; B) BGs/PLLA 10% wt membranes at different dilutions. Asterisks denote a significant difference compared with the reference, * $P < 0.01$, ** $P < 0.001$.

In vitro biological tests showed a reduction of the metabolic activity of cells when PLLA/BG composites are employed. These findings suggest that a high amount of polymer (8 %wt) reduces the effect of 10% wt BG powders on the cell activity, as discussed previously in Figure 5.14. Although these results do not seem to be promising, the use of PLLA/BG composites might allow one to overcome a problem related to the cytotoxic effect of bioactive glass shown at high concentrations, as widely discussed in literature ((Q. Fu et al. 2010)). In fact, the most evident finding of the biological analysis is that the metabolism of ST2 cells cultured with dissolution product of composites exhibits more constraints than the pure BG powders.

5.3 Outline

In this chapter composite PLLA/BGs produced via TIPS and DIPS were presented. The integration of BG with two different composition (45S5 and 13-93) into scaffold produced via TIPS was investigated. The main problem addressed was the release of BG ions in aqueous solution. The BG pre-treated was the strategy to limit this release during the phase separation process. SEM micrographs showed that the BG45S5 interacted with the phase separation mechanism during the formation of the scaffold also if pre-treated. The results for BG13-93 revealed that pre-treatment helps the integration process, since the BG composition influences deeply the finally morphology of composites. The different behaviour could be related to the higher reactivity of 45S5 respect to 1393. Furthermore, the pre-treatment does not alter the bioactivity of BG13-93, which remains unaffected and the problem of brittleness has been partially overcome. Moreover, the combination of HA/BG13-93 pre-treated gives the scaffold a morphological feature homogeneous enough and make it less brittle. An increase in mechanical strength was observed at 5% wt concentration of BG13-93 pretreated. Nevertheless, studies to deeply understand the role of bioglass in the crystallization processes typical of liquid/liquid separations are still ongoing.

In the DIPS technique used to produced composite membrane, BG do not interfere with the phase separation process, indeed the amount was increase until 10%wt. It is known that pure polymers lack biological activity and osteoinduction, which justifies the need for loading the polymers with fillers (Dehnavi et al. 2018)(Nasajpour et al. 2018)(Pitaluga et al. 2018)(Bejarano et al. 2016)) or growth factors (Zigdon-Giladi et al.

2017). BG inorganic substances were combined with a polymer in order to enhance biological performance of PLLA. The basic cellular functions such as metabolic activity, proliferation and VEGF secretion vary largely depending on the concentration and type of bioactive glass in the dissolution products. It was observed that the IDPs of PLLA/BG13-93-B3 at the highest concentration (10 %wt) stimulate the cells to secrete the VEGF. The incorporation of BG particles into polymer-based composites enhances the bioactivity of pure PLLA already after 14 days. *In vitro* mineralization test proved the prominent role of BG particles in the development of carbonated hydroxyapatite (HCA) in SBF. In the future a direct test should be designed in order to investigate the interaction of cells with the membrane surface. Nevertheless, further investigations will still be necessary to investigate how the osteoinduction and angiogenic potential of composites vary for filler concentration exceeding of 10 %wt. The optimal BG quantity will be determined when a fair compromise between good bioactivity and satisfactory angiogenic potential of composite is achieved.

Chapter 6

6 Composite scaffolds PLLA-HA-BG

In this chapter PLLA-HA-BGs scaffolds produced via TIPS are proposed as substitutes to repair the bone tissue. Previous studies reported that the combination of polymer-hydroxyapatite-bioglass allows one to fabricate scaffold with better mechanical and structural properties (Motealleh et al. 2017) but also to increase their biocompatibility. Furthermore, simultaneous coating of HA and BG on polymeric scaffold induced a higher degree of bone regeneration (Dinarvand et al. 2011).

The effect of the simultaneous combination of polymer with hydroxyapatite and bioglass will be here investigated, with the aim of improving mechanical properties and biological activity compared to those of polymeric matrix alone. *In vitro* biodegradation, bioactivity studies were performed in the labs of Biomaterials Institute, Friedrich-Alexander University, Erlangen-Nuremberg (Germany) under the supervision of my cotutor, prof. Aldo Boccaccini.

6.1 Scaffold preparation

The protocol followed for composite scaffold production was analogous to that adopted for PLLA/HA or PLLA/BG scaffolds, described in the previous chapters. The fillers employed were hydroxyapatite by Alhenia (Camceram III, HA coating powder) and bioactive glass 13-93 with composition (%wt) SiO₂ 53%, Na₂O 6%, CaO 20%, P₂O₅ 4%, K₂O 12%, MgO 5%. TIPS protocol followed for scaffold production was similar to that

reported in 4.1.1 paragraph (temperature 30°C, residence time 30 minutes). The solution had a PLLA concentration of 6 wt% with a dioxane/water weight ratio of 87/13 and an appropriate amount of filler. Several samples were prepared with a PLLA/filler weight ratio of 95/5 at three different HA/BG13-93 ratios (100/0, 90/10, 80/20). For the various characterizations, the samples were opportunely cut with different shapes.

6.2 Scaffold characterization

Several traditional techniques were employed for samples characterization, following the protocol described in chapter 3.

SEM analysis were carried out to observe the microstructure and bulk morphology of porous composite scaffolds.

The porosity of PLLA, PLLA-HA, PLLA-HA-BG scaffold was determined by gas pycnometer on several samples (n=5).

Mechanical properties of scaffolds were determined by compression test as illustrated in 3.1.5 paragraph. At least five disc-shaped specimens (diameter 13 mm, thickness 3 mm) were tested for each sample.

6.2.1 Differential scanning calorimetry analysis

Differential scanning calorimetry (DSC) was used for characterization of thermal properties of the scaffolds (n = 3). Melting temperatures and melting enthalpies of the samples were evaluated by thermograms obtained with a Setaram 131 evo Differential Scanning Calorimeter (DSC). The samples, after an initial stabilization at 30 °C for 15

min, were heated from 30 to 210 °C at a rate of 10 °C/min, under a nitrogen gas flow of 1 ml/min. The degree of crystallinity (χ_c) was estimated using the enthalpy of melting change according to the equation (Dziadek et al. 2015): $\chi_c = \Delta H_m / \Delta H_m^0 * 100\%$, where ΔH_m^0 is the enthalpy of melting (93 J /g) for 100% crystalline PLLA (Diaz et al. 2014).

6.2.2 In vitro bioactivity test

According to the protocol illustrated in 3.1.8 paragraph, composites bioactivity was evaluated by immersion in a simulated body fluid (SBF). Samples were cut in parallelepiped shape (10 x 5 x 2 mm) and soaked in 12 ml of SBF. The formation of HCA layer was investigated at five time points up to 28 days.

6.5 Results

A homogeneous filler distribution inside the polymeric structure can be observed by looking at SEM micrographs (Figure 6.1). The TIPS thermal path followed in this work brought to an average pores size of $60 \pm 1.5 \mu\text{m}$ for pure PLLA and PLLA-HA, whereas the average pore size of PLLA-HA-BG samples increases up to $100 \pm 2.3 \mu\text{m}$. That different behavior, currently under study, needs to be fully understood and clarified. However, microscopy analysis of each sample has indicated the existence of a highly porous structure with very good pore interconnectivity.

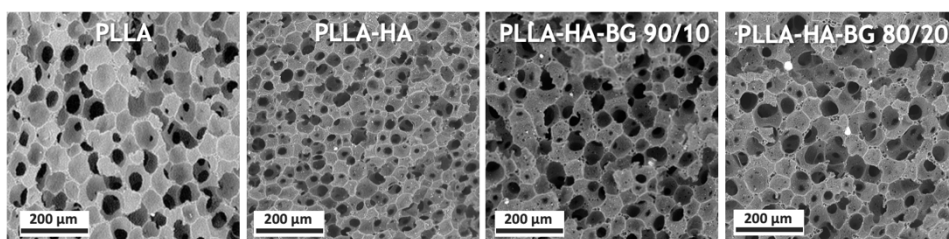


Figure 6.1 SEM micrographs of complete set of scaffolds as prepared via TIPS

As reported previously, the higher porosity was more biologically beneficial for porous scaffolds since it facilitates cell migration, blood circulation and vascularization (Shavandi et al. 2015). The scaffolds porosity has been estimated using a gas pycnometer, and results are displayed in Figure 6.2. As it can be observed, the porosity was $> 90\%$ for all scaffolds. Combining HA-BG 90-10 the porosity obtained was slightly lower. A high SD was observed for composite scaffold than pure PLLA. slight variation of micro-porosity from sample to sample could be due to filler that could be a nucleation centre during the TIPS process.

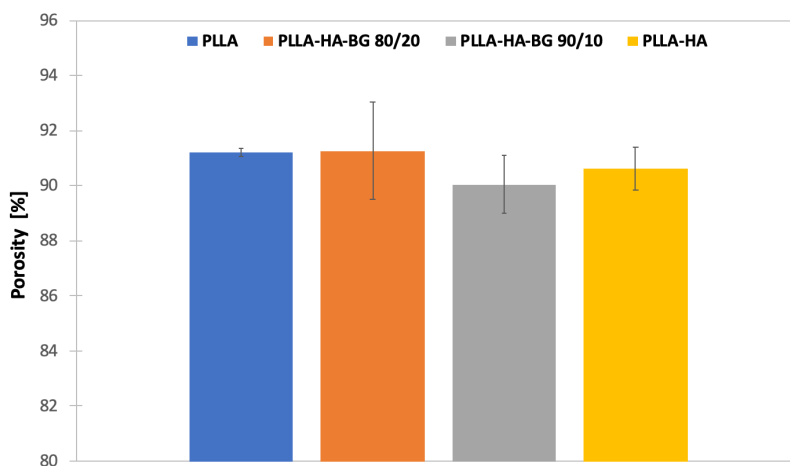


Figure 6.2 Porosity evaluation of composite scaffolds PLLA-HA, PLLA-HA-BG 90/10, PLLA-HA-BG 80/20. Scaffold in pure PLLA was used as control.

The effect of BG and HA incorporation into polymer matrix on the degree of crystallinity and melting temperature of PLLA was studied by DSC analysis. Figure 6.3 reports the crystallinity degree (χ_c) and the melting temperatures (T_m) of the composite scaffolds. The degree of crystallinity of all composite scaffolds slightly decreased compared to pure polymer. This effect was more evident in PLLA-HA-BG scaffolds and could be attributed to simultaneous effect of HA and BG particles. As a matter of fact, it has been reported that BG promotes the crystallization of the PLLA matrix, considering the particles acting as heterogeneous nucleating agents (Barroca et al. 2010). On the other hand, PLLA/HA samples exhibited a decrease in the crystallinity due to HA particles

amount which boost the rigidity of the polymer chains, according to Diaz et al. 2014. It may be argued that in presence of HA, BG particles do not play any nucleating effect but their contribution to the rigidity of polymer chains helps in further reducing final crystallinity. The melting temperature of PLLA-HA composite materials remained practically unchanged. Furthermore, a slight decrease in the melt peak from around 181 °C to 178 °C and 176 °C for PLLA-HA-BG 90/10 and PLLA-HA-BG 80/20. It seems that increasing the amount of BG the materials became more amorphous, so the temperate was reduced, in line with the observation reported above concerning the degree of crystallinity.

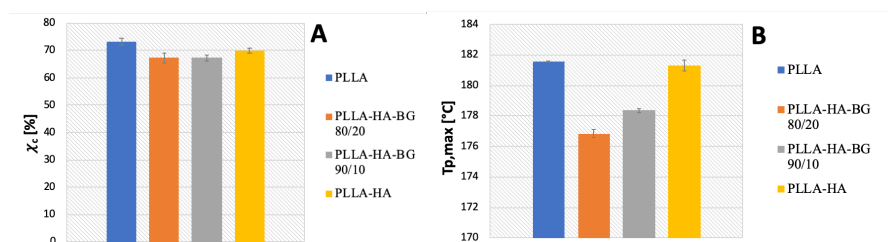


Figure 6.3 Thermal properties of PLLA, PLLA/HA, PLLA/HA/BG 90/10 and PLLA/HA/BG 80/20 scaffolds. (A) crystallinity (%); (B) melting temperature (°C).

The mechanical properties also played an important role in the cell behaviour. The scaffold, as a biomimetic structure of extracellular matrix, needs a certain level of mechanical strength to provide enough structural stability to support cell attachment (Mao et al. 2018). Young's modulus has been calculated from the linear region of the stress-strain curve and is displayed in Figure 6.4. All scaffolds filled with BG/HA

particles showed lower compressive modulus comparing to the PLLA only. Scaffold in pure PLLA shows the highest modulus of 9.3 ± 1.1 MPa. The modulus of PLLA- HA-BG 90-10 group was 4.8 ± 1.2 MPa, significantly lower among all groups ($p < 0.001$). The result may be due to the fact that mechanical properties of PLLA are superior to those of BG, which would fail prematurely under compressive load for its brittleness. PLLA/HA-BG 80-20 scaffolds possess significantly low stiffness ($p < 0.001$) with modulus of 5.8 ± 0.7 MPa while PLLA/HA of 7.5 ± 1.2 MPa ($p < 0.05$). These results are in accord with recent studies indicating that adding of BG reduces the stiffness of the composite matrix or filled with only HA (Gao et al. 2014).

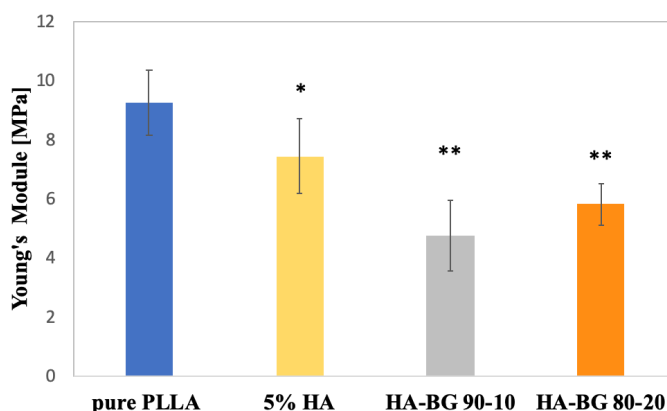


Figure 6.4 Young's modulus of different scaffold. Significant difference between each composite with the neat PLLA (*) $p < 0.05$, (**) $p < 0.001$

In vitro bioactivity test

The ability of the different composite scaffolds to form a HA-like surface layer when immersed in SBF is highlighted by SEM micrographs (Figure 6.5), FTIR spectra and XRD. For the sake of brevity only results concerning 28 days are here discussed, being representative of all samples.

FTIR patterns of all samples, before and after SBF treatment, are displayed in Figure 6.6. Before treatment, all the spectra are essentially identical, probably due to very low filler concentration, making the bands of PLLA overlapping each other. Instead, peaks related of HCA appear at 1460 and 1420 cm^{-1} as CO_2 replaced sectional PO_4^{3-} . Moreover, the displacement of the carbonyl signal from 1746 cm^{-1} to 1755 cm^{-1} is attributed to interaction of hydrogen bond of carbonyl group of PLLA and the surface P-OH groups of HA (Zhou et al. 2007).

These few evidences support the hypothesis of a HCA-like layer formation in composite scaffold upon immersion in SBF, even if only in a small regions. X-ray diffraction analysis of investigated systems has confirmed in situ formation of hydroxyapatite within composite scaffold, as depicted in Figure 6.7. The crystallinity of PLLA was weakly influenced by the presence of BG, due to its low BG. Furthermore, there is no presence of peaks of other molecular structures. After 28 days in SBF, in the composite scaffold the peaks corresponding to the polymer were still present but the peaks centered at $2\theta = 35^\circ$, typical of HA is clearly identifiable, even though it was not possible to distinguish whether it is the HA filled in the PLLA during the preparation process. The scaffolds surface appears however not completely bioactive as observed in SEM micrographs where the formation of HCA is visible in few points of the surface. These results

corroborate the hypothesis of the formation of a HCA-like layer in all samples upon immersion in SBF. This effect is more evident in scaffolds PLLA-HA-BG1393 80/20. Indeed, a greater amount of bioactive glass within the scaffold is shown to improve its bioactivity.

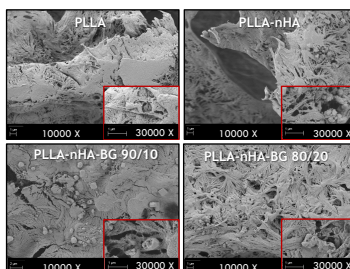


Figure 6.5 SEM micrographs of all samples after treatment in SBF for 28 days

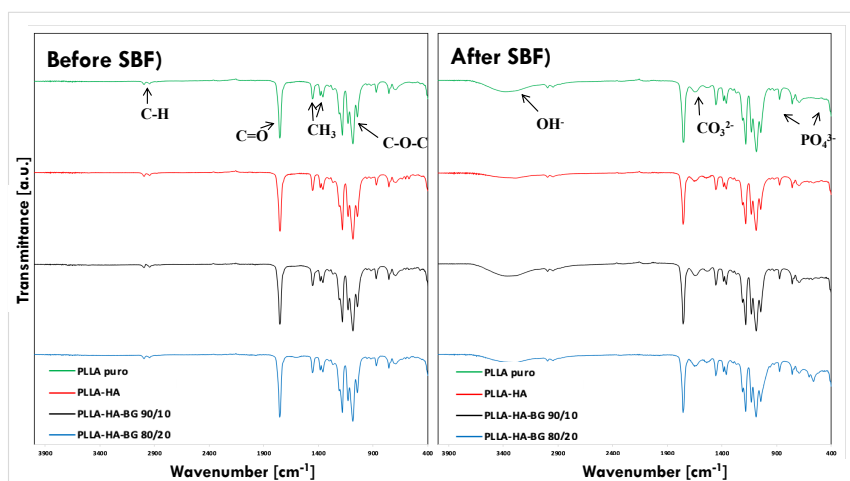


Figure 6.6 FTIR spectra of all samples, before and after treatment in SBF for 28 days

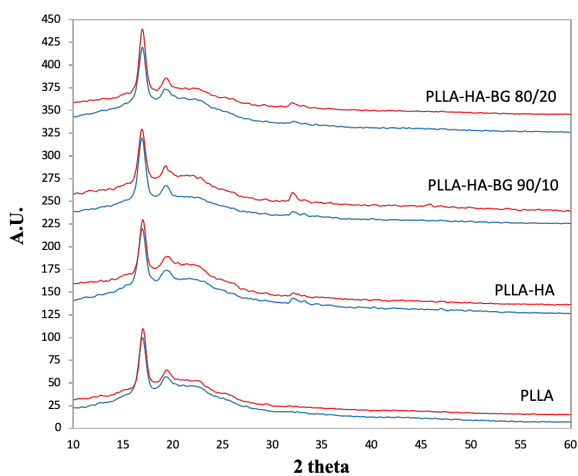


Figure 6.7 XRD patten of all samples, before (blue) and after treatment in SBF for 28 days (red).

In summary, the combination of HA and BG 13-93 as fillers produces scaffolds with homogeneous morphological structure and makes them less brittle than scaffolds with BG alone. However, due to the low bioactivity of HA alone, a strategy to increase the amount of BG to incorporate in the polymer should be explored.

Chapter 7

7 Scaffold for Ligaments regeneration

The ideal scaffold for ligament tissue engineering should be biocompatible and possess mechanical and functional characteristics comparable to the native anterior cruciate ligaments (ACL). Optimization of seeding methods is key to obtain an engineered ligament tissue with a histological structure *in vitro* analogous to native tissue. Seeding cells efficiently and uniformly onto three-dimensional scaffolds allows cells to cooperate positively with biomaterials (Lv et al. 2016). Several techniques, including the cell suspension seeding (Fan et al. 2008), fibrin gel (Lu et al. 2005; Rigogliuso et al. 2014) and spheroids (Hoyer et al. 2014) seeding methods, have been used to seed cells on scaffolds.

In this chapter, a new approach in ACL regeneration is proposed focussing on the development of a simple and quick technique for *in vitro* cell seeding of lapine ACL fibroblasts into tubular-shaped scaffolds produced performing a Diffused-Induced-Phase Separation (DIPS) process and supplemented or not with bioactive glass, BG13-93. The possibility to combine different processing strategies, like DIPS and electrospinning (ES), was investigated afterwards, so can improve the biomimetic degree of engineered scaffold as well as their physical properties. The tubular scaffolds were produced at the University of Palermo and *in vitro* biological tests

were carried out in Prof. Gundula Schulze-Tanzil's laboratory at the Anatomy Institute of the Paracelsus Medical University in Nuremberg.

7.1 Composite tubular scaffold

7.1.1 Scaffold preparation

Tubular scaffold in poly-lactic-acid (PLLA) and bioactive glass (BG13-93) were obtained via DIPS, after a dip coating around a cylindrical support in steel inox AISI 316, followed by a diffusion induced phase separation process. All steps of DIPS process were described in the Chapter 5, but here they are summed up briefly. First of all, PLLA/dioxane solution 8 %wt in polymer was prepared and 5%wt of BG-1393 was added but to increase the particles dispersion inside, the solution was sonicated for 30 min. Two different supports, $d=1.2$ mm and $D=2$ mm as diameter, were immersed into solution (dip-coating bath) at a temperature of 45 °C and were slowly pulled out at a constant rate of 16 cm/min. Then the bar was pool-immersed into a second bath (DIPS or coagulation bath), containing pure water at the same temperature, for 10 minutes. At the end the scaffold was extracted from the bath, rinsed in distilled water, and dried at room temperature. Tubular scaffolds as obtained with diameter $d=1.2$ mm (small tube_ S_{mt}) and 2 mm (big tube_ B_{it}) and 4 cm long were opportunely cut to carry out the next characterization tests. Tubular scaffolds in neat PLLA with the same shape and dimension were used as control in

each trial.

7.1.2 Scaffold characterisation

Scaffold microstructure was detected by SEM analysis as previously described in 3.1.2 paragraph. For estimating the pore size in the inner surface and the wall thickness, several pictures (n=10) were taken. For each one, the average pores size was calculated and the values were reported as mean value $\pm$ standard deviation.

The inner structure, pores size, wall thickness and BG distribution were evaluated by micro-computerized tomography (μ CT). The X-ray scans were acquired in high-resolution mode and images with a pixel size of 1.25 μ m were obtained. A quantitative analysis to estimate the amount of BG was carried-out following the protocol described for PLLA-HA scaffold (chapter 4).

Mechanical stability of tubular scaffolds w/wo bioglass was examined by dynamometer (Instron model 3365). Tubes were properly fixed in the grips and permitted to elongate, using an extension speed of 1 mm/min. The tests were performed on tubes fixed to the pneumatic jaws of the dynamometer. The thickness of each sample was measured before test whereas the gauge length was 20 mm for all the samples. Elastic modulus (E), stress strength and elongation at break (ϵ) were determined. Seven samples were tested for each material and the average values of the mechanical parameters were reported with their standard deviations.

7.1.3 In vitro cell culture

Lapine ACL fibroblasts cells (LKB) were cultured in T175 culture flasks with growth medium (Ham's F-12/Dulbecco's Modified Eagle's (DMEM) Medium 1:1) containing 10% fetal calf serum (FCS), 1% (v/v) penicillin/streptomycin, 2.5 µg/mL amphotericin B, 1% (v/v) MEM-amino acids, and 25 µg/mL ascorbic acid at 37 °C and 5% CO₂. The growth medium was changed every 3 days in order to ensure the regular supply of nutrients to the cells.

Before the seeding on scaffold, the cells were counted using the trypan blue dye to exclude dead cells from the count.

7.1.4 Cell seeding protocols

First of all, scaffolds in PLLA/BG13-93 and neat PLLA were cut into pieces 2 cm long (Figure 7.1 A) and then sterilized in order to remove impurities, microbiological contaminants.

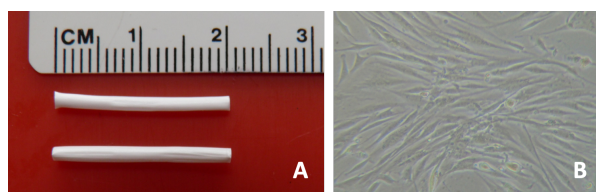


Figure 7.1 (A) Tubular scaffold 2 cm long for cell culture tests. (B) Lapine ACL fibroblasts monolayer before the seeding

Samples were soaked in ethanol 70% vol under vacuum and then placed in a mechanical shaker overnight. The day after, the specimens were washed with phosphate buffered saline solution (PBS) and pre-conditioned with cell culture medium for 24 h. Before the seeding, scaffolds dried on a sterile gauze to remove the medium into the tube.

In vitro cell culture trials were carried out by seeding ACL fibroblasts (Figure 7.1 B) inside the scaffolds following three different seeding strategies involving the use of (A) dynamic seeding with cells suspension, (B) fibrin gel and (C) spheroids. The different methods were compared in order to find the best way that allows to obtain a homogeneous fibroblast distribution inside the samples.

Based on scaffolds sizes, the volumes of culture medium required for seeding was calculated, such as to completely re-fill the tube without cells lost during the seeding. The volumes were estimated of 50 μ l for S_{mt}, 100 μ l for B_{it}. To guarantee a uniform distribution of cells into whole tubular scaffold, the seeding was made in two steps: half of cell suspension was inoculated on one side and half on the other side. Each protocol is described in details below.

Dynamic seeding with cell suspension (A protocol)

As mentioned in the literature, dynamic seeding can achieve better penetration and uniform distribution of the cells within the scaffold (Theodoridis et al. 2019). This protocol has been applied for inoculating cell suspension into the scaffolds. Two different concentrations were used: 1.6×10^4 cells/ μ l for small tube and 1.5×10^4

cells/ μl for big one. To promote cell adhesion, all scaffolds were incubated for 15 min, then rotated and maintained for another 15 min at 37 °C and 5% CO₂. Each sample was transferred into an Eppendorf tubes and soaked in 2 ml culture medium. Putting them into a bioreactor tube and allocating it on a roller mixer inside the incubator, a dynamic culture was simply executed. After 1 day of dynamic culture, the number of cells in the supernatant (not-attached on the scaffold) was calculated, estimating so the seeding efficiency.

Fibrin gel (protocol B)

ACL fibroblast cells were seeded using fibrin as a cell carrier. Cells were resuspended in a solution containing fibrinogen in culture medium prepared in a ratio 1:2 in volume. Immediately before seeding, 20 μl or 40 μl of thrombin (0.5 U/mL) were injected inside small or big scaffold, respectively. The cell-containing fibrin gel was injected onto the tubular scaffolds at the concentration of 800,000 cells/50 μl per small tube and 1,500,000 cells/100 μl per big tube, using micro-pipette. The samples were incubated at room temperature for 10 min in order to promote the polymerization process of the gel. After that the samples were transferred into petri dishes and the 2 ml medium were added.

Spheroid culture (protocol C)

The spheroids (three-dimensional self-assembled cell agglomerates) were prepared using the hanging drop method (Hahn et al. 2019; Neto et al. 2015), as illustrated in

Figure 7.2. In this technique, the cells tend to accumulate at the tip of the drop at the air-liquid interface, spontaneously aggregate, and finally form spheroids. However, drops volume should not be more than 50 μ l because the drop do not adhere to the petri dish (since the liquid surface tension is overcome by gravity) (Hoarau-Véchet et al. 2018).

The spheroids consisting of 50,000 cells per spheroid were harvested after 48 h.

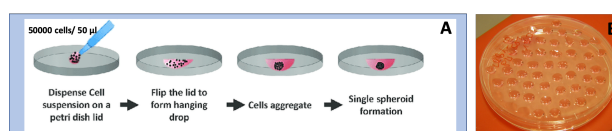


Figure 7.2 (A) Schematic representation of hanging drop methods; (B) All spheroids culture in drop in a petri dish. The drops were prepared suspending 50,000 cells/ 50 μ l of medium, adapted from Hoarau-Véchet et al. 2018.

The seeding protocol was carried out by inoculating spheroids into the specimen. The amount chosen were: 46 spheroids /50 μ l of medium into the small tubes and 88 spheroids/100 μ l of medium into the big tubes. All scaffolds were pre-incubated for 30 min and then a growth medium was added.

Regardless of adopted protocol, all scaffolds were cultured in petri dishes coated with 2 mL of growth medium, that it was changed 2–3 times a week. After well-defined culture times (1, 7 and 14 days), the scaffolds were extracted from medium and opportunely treated for further analysis.

7.1.5 Cell viability and morphology

Cell viability on scaffolds is essential for cellular compatibility and suitability in tissue engineering. Hence, cells morphology studies and viability tests were performed.

The staining solution for live/dead assay was prepared as reported in 3.2.1 paragraph. A drop of the FDA/PI staining solution was stilled on a thin glass slide and the samples were observed.

Cell morphology was investigated by H&E staining. First of all, the samples were fixed in 4% paraformaldehyde (PFA) solution at 4 °C. Subsequently the scaffolds were cut to obtain cross sections that were allocated into a histological cassette and fixed with istoKIT drops. The samples were subjected to dehydration and infiltration with paraffin at 60 °C and the paraffin block were prepared. Paraffin sections of 7 µm-thick for each samples were obtained and collected on glass slides that were stained according to traditional procedure.

Immunofluorescence staining

Immunolabeling was performed after 7 days of culture. In order to perform the staining, tubular scaffolds (1 cm length) were fixed at least for 1 day with a 4% PFA and stored at 4°C. Each tube was cut and small sections of 2 mm were obtained. They were washed with Tris buffered saline (TBS: 0.05 M Tris, 0.015 M NaCl, pH 7.6), overlaid with protease-free donkey serum (5% diluted in TBS with 0.1% Triton X-100 for cell permeabilization) for 20 minutes at room temperature. Then, scaffold

sections were incubated with primary antibodies type I collagen (produced in the goat) and vimentin (produced in the mouse) at 4°C overnight. Samples were rinsed with TBS before incubation with donkey-anti-mouse or goat-Alexa-488 and donkey-anti-mouse or goat-Cyanine3 (Cy3) coupled secondary antibodies, respectively, for 1 h at room temperature. Cell nuclei were counterstained using 4',6'-diamidino-2-phenylindol (1:100, DAPI, Roche, Mannheim, Germany) and Phalloidin-Alexa633 (1:50) to depict F-actin cytoskeletal actin architecture. The labelled cells were washed several times with TBS and the cross sections were examined by CLSM.

7.1.6 CyQuant proliferation assay

CyQuant is a fluorescent-based microplate assay which is able to determine the number of cells present in a sample. The working principle of this assay resides on the measurement of cellular DNA content via fluorescent dye binding. In fact, because DNA is tightly regulated, it can be assumed that each cell contains a constant amount of DNA and thus DNA content can be considered linearly proportional to cell number (L. J. Jones et al. 2001).

In order to prepare samples for the CyQuant assay, they were firstly frozen at -80 °C. A digestion step was first carried out: each scaffold was immersed in 300 µl TE buffer solution containing 7.5 mg/ml of Proteinase K, which is the enzyme which assures the destruction of cell membrane and the release of DNA. The day after, samples were maintained at -20 °C and after necessary time passed to freeze them, they were thawed: this freeze-thaw step was useful to facilitate the interaction between the CyQuant dye and

nucleic acids. The DNA was separated from the other sample component by a centrifugation step lasting 30 minutes; thereafter, DNA content was extracted, mixed with an equal amount of dye component and diluted with TE buffer solution (1:16 dilution factor) inside the microplate. In the same microplate, a serially diluted solution containing the DNA present in the kit was poured in order to construct the standard curve. After 1 hour of incubation in an oven set to 35 °C, finally, microplate was inserted into the microplate reader for the measurement of the fluorescence emission. DNA measurement was performed for all scaffolds at 1 day to define the seeding efficiency. Scaffold seeded with spheroids was repeated and also the test at 7 days was performed.

7.1.7 Results

Scaffold characterization

Some preliminary attempts to prepare scaffolds at various temperatures (30, 40 and 45 °C) were carried out at different support extraction rates (from 8 to 16 cm/min). Unfortunately, no homogeneous tubular scaffolds were successfully produced at 30 and 40 °C at all extraction rates tested. It was found that 45°C and rate of 16 cm/min were the better conditions to obtain wall thickness homogeneous in whole tube.

In Figure 7.3 the cross sections and wall thickness of pure PLLA tubular scaffolds were reported as example, since identical SEM micrographs were taken for the composite scaffold. Instead, the inner surface appears homogeneous both in pure PLLA, both in composite scaffold but with micropore size different Figure 7.3 A, B.

At same experimental conditions scaffolds with different diameter were obtained: d=1.2 mm with wall thickness of $78 \pm 1.2 \mu\text{m}$ (Figure 7.3 C, D); D=2 mm with wall of $80 \pm 1.5 \mu\text{m}$ (Figure 7.3 E, F). This morphology highlights how the different dimension of cylindrical support not influence the wall thickness, that depends significantly on the experimental parameters (Francesco Carfi Pavia et al. 2012). Prior studies have noted that changing the temperature and/or extraction rates, no homogeneous PLLA tubular scaffold were produced (La Carrubba, Pavia, and Brucato 2010). Morphological analysis of inner surface has revealed an open structure with micropores and an open porosity with interconnected pores was detected in the bulk of the wall.

This structure and the micro-porosity can be also appreciated in Figure 7.4, where scaffolds were stained with hematoxylin/eosin staining assay. As shown, the wall of the constructs presents micropores inside. This feature is fundamental for cell-scaffold interaction since it allows cells to penetrate into the surface and assuring a surface with more effective adhesion points for cells.

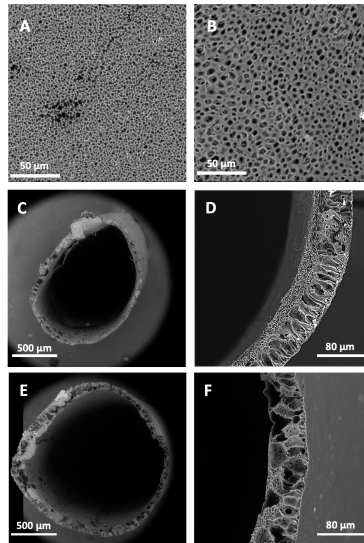


Figure 7.3 SEM micrographs of tubular scaffolds prepared with an extraction speed of 16 cm/min at 45°C. (A-B) Internal surface of pure PLLA and PLLA/BG13-93, respectively; (C-E) cross section of pure PLLA scaffold with diameters of 1.2 mm and 2 mm; (D-F) wall thickness of pure PLLA scaffold with diameter of 1.2 mm and 2 mm, respectively.

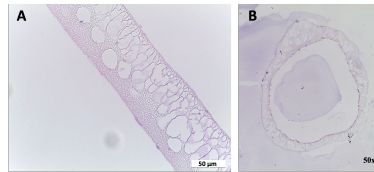


Figure 7.4 Hematoxylin/eosin staining of 7 μm thick sections of 1.2 mm PLLA. A) wall thickness; (B) cross section.

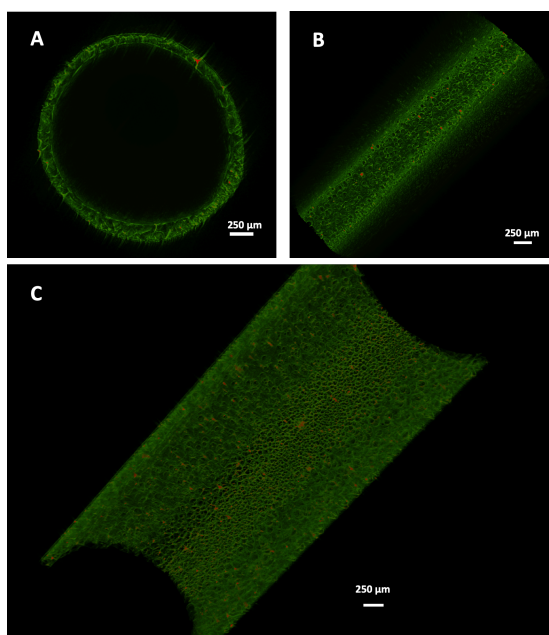


Figure 7.5 3D Reconstruction of 2mm PLLA/BG13-93 by CTVox software. (A) Cross section; (B) external surface; (C) inner surface. Polymer (green) and bioglass (red).

In Figure 7.5 a three-dimensional (3D) reconstruction of 2mm PLLA-BG was shown as representative sample. The 3D scaffold reconstruction confirmed the higher number of interconnected pores in tubular scaffold, but also a homogeneous distribution of BG powder in the whole sample (Figure 7.5 C). By quantitative analysis performed in three different VOI according to the protocol described in paragraph 4.1.2, the amount of BG

filler into the scaffold was 4.6 ± 0.2 %. This value is comparable with the nominal value, so could be affirmed that during the fabrication process BG particles are not lost. In the graph below (Figure 7.6), the mean pores size in the inner surface is reported for pure PLLA and PLLA/BG13-13 scaffold. A significant difference ($p < 0.01$) between two samples was noticed. Especially, pure PLLA shows micropores of 3.5 ± 1.7 μm , while PLLA/BG of 6.1 ± 2.9 μm . It is easy to notice that, with respect to pure PLLA scaffolds, the pores size in the composite is major. A possible explanation for this might be that the BG accelerates locally the diffusion process of the solvent in the non-solvent, leading to the formation of a porous structure with larger pores.

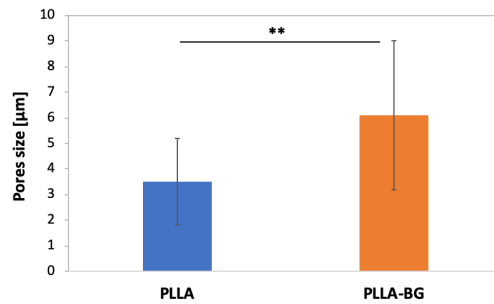


Figure 7.6 Comparison between pores size [μm] in the inner surface of pure PLLA and PLLA/BG13-93 tubular scaffold. Values are reported as mean value $\pm$ SD ($n=10$). Asterisks denote a significant difference between two groups (** $P < 0.01$).

In Table 7.1 the values of the elastic modulus, tensile strength, as well as the elongation at break of the PLLA and composite tubular scaffolds with different diameters are

summarized. The average modulus of small PLLA was the higher, 564.7 ± 24 MPa, while the value lower was 342.3 ± 27.9 for big PLLA-BG. It was observed that pure PLLA had the highest rate of elongation (ε) with 36.11 ± 6.7 and 30.0 ± 7.1 , respectively for small and big tubes. Looking at the tensile strength (TS), it can be seen that TS value decreased by the addition of BGs and this hovered between 8–7 MPa for PLLA-BG. The trend for elongation at break was equivalent to the tensile strength.

One unanticipated finding was that elastic modulus of composite decrease in the composite tubes. Usually, this value increase in composite since the nature of BG which is stiffer than PLLA. This result may be explained by the fact that the presence of bigger pores in the PLLA-BG scaffold, as observed by SEM images, reduced elastic modulus in comparison to pure PLLA, as also previously reported by others (Dehnavi et al. 2018). The tensile strength and elongation decreased due to the addition of the BG powders in the PLLA matrix (Sunandhakumari, Vidhyadharan, and Alim 2018). Being BG a rigid inorganic material, when the composites are stretched BG could rupture the PCL polymer as supporting points without deformation ability followed by the reduced elongation at break. On the contrary, the pure membranes indicated the substantial elongation at break, which is prevalent among ductile thermoplastic polymers. From comparing all scaffolds it can be possibly appreciated that the mechanical properties of big PLLA are comparable with the small PLLA-BG scaffolds. These results match with those observed in earlier studies and they highlight that mechanical properties depending on the materials

composition, dimension, filler concentration and porosity (Li et al. 2015; Lopresti et al. 2020; Rogina et al. 2016).

Table 7.1 Mechanical properties of pure PLLA and PLLA-BG13-93 tubular scaffold with different diameters.

Property	PLLA small	PLLA big	PLLA_BG13-93 small	PLLA_BG13-93 big
Diameter (mm)	1.2	2	1.2	2
Wall thickness (μm)	78 ± 1.2	76 ± 1.5	78 ± 1.2	76 ± 1.5
Length (mm)	20	20	20	20
Avg. pore size (μm)	3.5 ± 1.7	3.5 ± 1.7	6.1 ± 2.9	6.1 ± 2.9
Young's Modulus (MPa)	564.7 ± 24	498.7 ± 18.5	379.4 ± 27	342.3 ± 27.9
Tensile strength (MPa)	8.96 ± 0.89	8.12 ± 0.86	8.02 ± 0.31	7.19 ± 0.78
Strain at break (%)	36.11 ± 6.7	30.0 ± 7.1	30.2 ± 11.6	9.88 ± 2.9

Comparison between seeding protocol

One of the problems that have to be overcome in tissue engineering is the cell seeding efficiency. As a matter of fact, during the early stages of seeding, the injected cells tend to come out from the scaffold toward the culture plate; moreover, above all in static culture, the cells start to colonize the scaffold to some degree and later they start to

migrate colonizing the entire circumference of the tube, thus leading to a slow and irregular colonization of the luminal surface of the scaffold (Rigogliuso et al. 2014). For this reason, the main purpose was seeking a solution to obtain the best result in term of complete scaffold colonization in a short time. Different seeding protocols were compared. Life/dead assays, Hematoxylin/Eosin and immunofluorescence staining were employed to verify cells viability, adhesion to the polymeric structure and migration into the scaffold. Cell proliferation was calculated from DNA content using CyQuant assay.

Live/dead assay

Dynamic seeding using a cell suspension (protocol A)

For this protocol the seeding efficiency was determined as described above. The numbers of cells attached to the scaffold surface was estimated from the difference between the cell number seeded and that counted in the medium after the 24 hours of dynamic seeding. For all scaffolds the results were collected in the Table 7.2.

Table 7.2 Percentage of attached cells in all scaffolds, after 24 h of dynamic seeding

	<i>PLLA</i>		<i>PLLA-BG13-93</i>	
Diameter (mm)	1.2	2	1.2	2
n° cells in medium	180,000	382,500	195,000	135,000
attached cells (%)	78	74.5	87	91

It can be observed that in composite scaffolds the percentage of cells attached to the surface was always higher than in neat PLLA, regardless of tube diameter. In accordance

with the present result, previous studies have demonstrated that the BG favored osteoblast cell attachment (Brown et al. 2008), increased attachment and viability of the fibroblasts (Nokhasteh et al. 2018).

Live/dead staining was used to prove cell survival within the four different scaffolds. Fluorescence micrographs via CSLM of cross sections (Figure 7.7) were taken after 1, 7 and 14 days of culture. The vitality assay showed that the majority of the fibroblasts were still alive within all tubes during the two weeks of observation time. At day 14, an even larger area of the lumen of the tube was covered with viable cells in all scaffold types. Longitudinal sections of the small tubes of pure PLLA after 1 and 7 days of culture are reported in Figure 7.11 A, B, as an example. It could also be noticed by Figure 7.11 A that ACL fibroblasts rapidly attached (1 day) and spread over the inner surface of the tubes. At 7-day (Figure 7.11 B), the inner surface of tubular scaffold was covered by a large number of cells, with mostly live cells (green cytoplasmic fluorescence) and few dead cells (red nuclear fluorescence). The confocal images showed the elongated shape of the cells highlighting a good cytocompatibility of the materials.

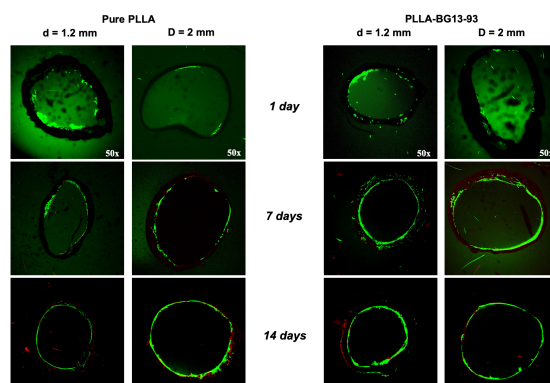


Figure 7.7 Cross section of all scaffolds stained with live/dead assay after 1, 7, 14 days of culture. Alive cells are visible in green colour, whereas dead cells are the red dots. Magnification: 50x.

Fibrin gel (protocol B)

Fibrin protocol was done by suspending the cells in a fibrin gel polymerized within the tubes by using of thrombin. Live/dead staining pictures are displayed in Figure 7.8. Here only the images at 1 and 14 days are reported because any different was found between 1 and 7 day. Figure 7.8 shows that, after 48 hours from the seeding, cells remained captured inside the fibrin gel, anyway after 14 days the cells migrate from the fibrin to the tube surface colonizing the lumen of tube, especially they penetrate into the wall. This effect was clearly shown in the composite scaffolds.

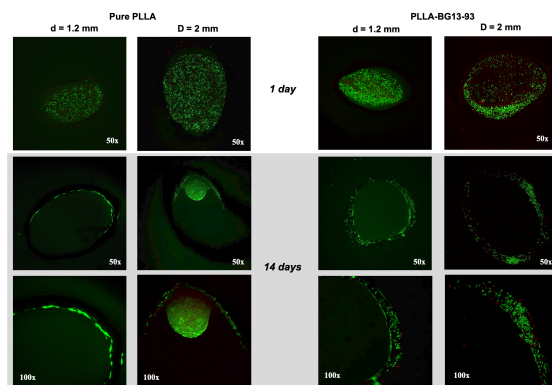


Figure 7.8 Cross section of all scaffolds stained with live/dead assay after 1 and 14 days of culture. Alive cells are visible in green colour, whereas dead cells are the red dots. Pictures at two magnifications are shown at 14 days.

The result at 1 day was confirmed by previous work. With this approach, no cell suspension was lost during the seeding process because the fibrin rapidly polymerizes and the gel remained into the tube. It further ensures homogeneous cell distribution throughout the scaffold (Mol et al. 2005; Stekelenburg et al. 2009).

The Figure 7.11 C, D presents of the longitudinal section of 1.2 mm PLLA-BG13-93 scaffold. At the 1 day, the cells have a round shape and they are immersed in a fibrin gel, as previously observed in the cross section (Figure 7.11 C). Instead at 14 days ACL appear stretched and migrated to the tube surface forming cell layer (Figure 7.11 D). On

the contrary to protocol A, the cells were attached on the surface but in a random way without orientation.

Spheroid culture (protocol C)

After resuspending the spheroids in the cell culture medium, a live/dead staining where carried out to be sure that before the seeding the spheroids were live.

The Figure 7.9 A displayed the spheroids with diameter of $\approx 380 \mu\text{m}$ before the seeding. All cells of the 3D agglomerate are live. In Figure 7.9 B a spheroid was shown that left during the seeding the scaffold and adhered to the petri dish showing emigration of cells.

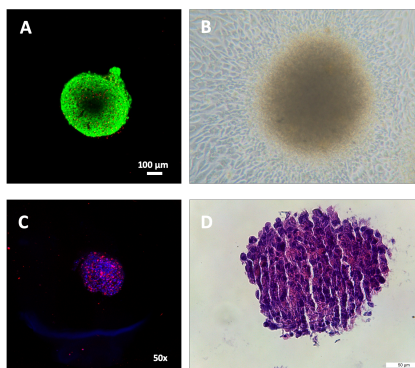


Figure 7.9 Spheroids of ACL fibroblast. (A) Live/dead staining before seeding; (B) control in plate after 7 days from the seeding; (C) Vimentin immunolabeling (red) and DAPI (blue) after 7 days (D) H&E staining after 7 days of culture in a scaffold.

Figure 7.10 compares the pictures of the live/dead staining assay performed on the surfaces of PLLA and PLLA/BG13-93 scaffolds with different diameter. The pictures at the first day highlight that the surface of the tube was not homogeneously colonized, since spheroids were identified only in few spots. After 14 days cells migrated and appeared stretched on the scaffold surface but did not completely cover it. This finding was also confirmed by pictures of longitudinal sections (Figure 7.11). In fact, at the first day some spheroids surrounded by few stretched cells migrated onto the scaffold surface can be observed Figure 7.11 E. At 14 days most of the cell nuclei were oriented in a direction parallel to the channels (Figure 7.11 F).

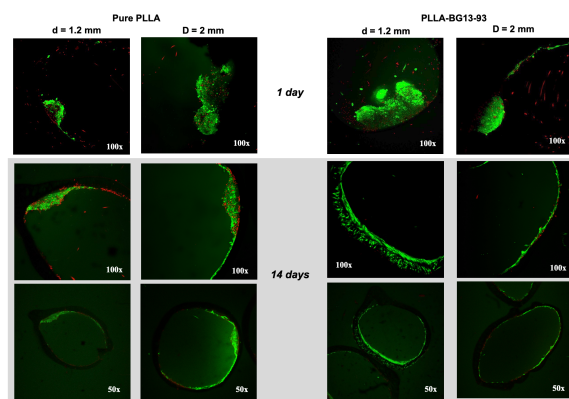


Figure 7.10 Cross section of all scaffolds stained with live/dead assay after 1 and 14 days of culture (Protocol C). Pictures at two magnifications are shown at 14 days.

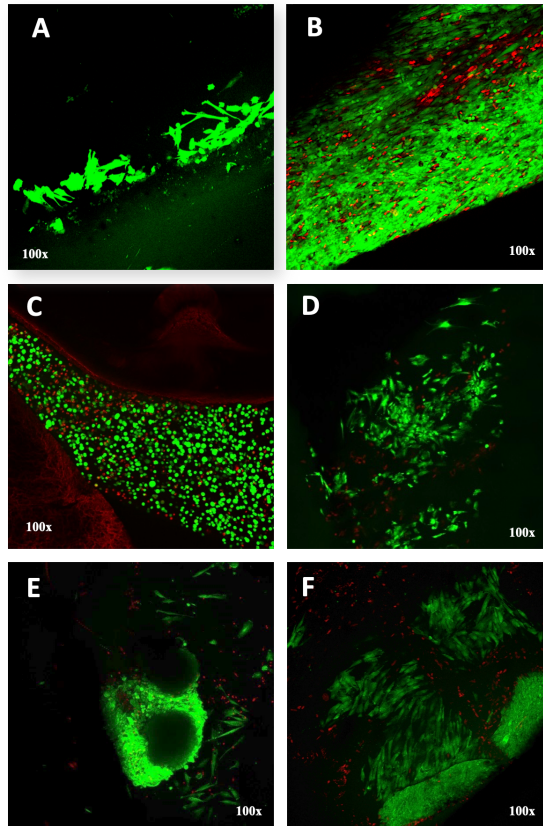


Figure 7.11 Longitudinal section of PLLA-BG13-93 d=1.2 mm scaffolds stained with live/dead assay after 1 day (A-C-E) for protocol A, B and C, respectively. (B-D-F) cells at 14 days of culture following protocol A, B, C respectively. Alive cells are visible in green colour, whereas dead cells are the red dots. Magnification: 100x

Haematoxylin/Eosin histologic assay

The Hematoxylin/eosin (H&E) staining didn't give useful information about the morphology of cells stretched on the surface of tubes. The main problem has been the dimension of the samples (too small). Sometimes, the cutting was so difficult that sometimes the samples were destroyed.

In the Figure 7.12 the H&E stained cross sections of 1.2 mm PLLA scaffolds seeded following two different protocol: A and B are compared. It can be observed that in protocol A, the cells (purple colored) adhered to the inner surface after 7 days. On the contrary adopting the protocol B, the cells are encapsulated in the fibrin gel at 7 days, yet. This evidence is in agreement with the information deduced from the live/dead pictures. Elongated nuclei were also observed within the fibrin (Figure 7.12 B-D). This is a typical fibroblasts morphology.

As representative example, the H&E stained sections of 2 mm PLLA-BG13-93 seeded with spheroids are reported comparing results of 1 and 14 days (Figure 7.13). At 1 day, the picture shows that the spheroid has been detached from the surface of the scaffold due to the sample preparation for the staining. Indeed, after 14 days (Figure 7.13 B, D) the spheroids appeared attached and slightly extended at the wall. This finding is consistent with that of live/dead staining: there occurred cell migration from the spheroids to the surface because the cells attempted to colonize the complete tubular surface.

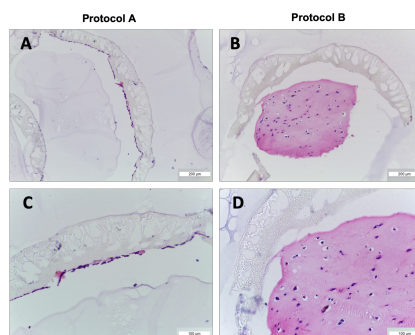


Figure 7.12 Day 7 hematoxylin/eosin staining assay of sections of PLLA $d=1.2$ mm scaffolds seeded with: (A-C) cell suspension according to protocol A; (B-D) fibrin gel mediated seeding (protocol B). Magnification: 100x, 200x.

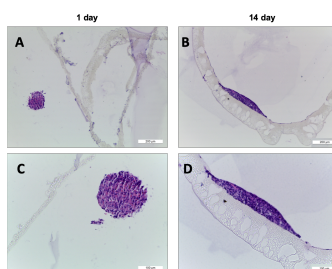


Figure 7.13 Hematoxylin/eosin staining of PLLA-BG $D=2$ mm scaffolds seeded with spheroids (protocol C) after 1 (A-C) and 14 days (B-D). Magnification: 100x, 200x.

Immunofluorescence staining

In order to detect cell morphology and extracellular matrix deposition, the fibroblasts cell seeded into the scaffold were stained for a cytoplasmic intermediate filament protein,

vimentin and type I collagen, the main component of the ligament extracellular matrix (ECM). The nuclei were stained in blue with DAPI. The confocal micrographs were taken at 7 days of culture for all samples and for all seeding protocols. Here, images of the samples 1.2 mm PLLA-BG13-93 seeded following three protocols are shown as representative of all samples.

Immunofluorescence staining demonstrated that cells are uniformly distributed along the tube, maintain elongated morphology, and have the capacity to synthesize ECM (Figure 7.14 C, D). This is particularly noticeable in scaffold seeded with protocol A and it is consistent with data obtained by live/dead staining (Figure 7.11 B). With the protocol B, at 7 days the cells were immersed into the fibrin and only the cell nuclei in blue were well highlighted (Figure 7.15) since the fibrin interfered with the staining.

The expression of type I collagen was difficult to detected. In particular the polymer absorbs the dye and sometimes does not allow to individuate the collagen expressed by the cells. In this Figure 7.16 type I collagen (green) and vimentin staining is reported. Assessment of type I collagen and vimentin was qualitative and was based on evaluation of their abundance around and between the cells within the surface of tubes. In particular an accumulation of cell nuclei was detected in the spheroids.

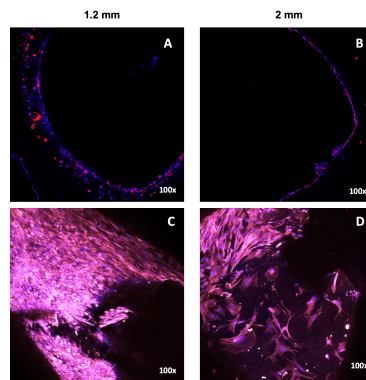


Figure 7.14: Immunolabelings of PLLA/BG13-93 scaffold seeded following protocol A (cell suspension). Cell nuclei are stained in blue and vimentin in red. (A-C) Cross and longitudinal section of a small scaffold, (B-D) cross and longitudinal section of a big scaffold.

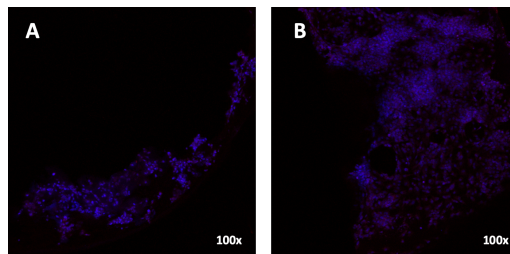


Figure 7.15 : Immunolabelings of 1.2 mm pure PLLA-BG13-93 scaffold with fibrin gel 7 days after seeding: cell nuclei (blue) and vimentin (red). Magnification 100x.

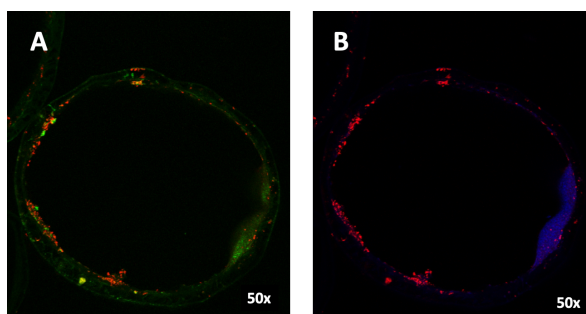


Figure 7.16: Immunolabelings of 1.2 mm pure PLLA-BG13-93 scaffold with spheroids 7 days after seeding: (A) cell nuclei and vimentin; (B) collagen type I (green) and vimentin. Magnification 50x.

CyQuant proliferation assay

In order to detect which seeding protocols show greater efficiency, the CyQuant proliferation assay was performed at 1 day. All results were collected in Figure 7.17. They are presented as a concentration expressed in cell number/scaffold. Comparing all seeding protocols, it can be observed that the protocol A and B showed the higher cell numbers per scaffold. On the contrary, the protocol C showed a significantly decreasing number of cells attached than protocol A ($p < 0.001$). Given the difficulty of the seeding technique, a lower number of cells attached on the scaffold surface was found. The result was in agreement with staining test, and it confirms that only few spheroids were attached to the scaffold while the others were lost during seeding. It was more evident in scaffolds with 2 mm as diameter. The cell number in the PLLA scaffold seeded with protocol A was analogous to that with the protocol B, both in the small tube ($p < 0.001$)

and in the big one ($p < 0.05$). On the contrary, the cell numbers seeded on PLLA-BG13-93 following the protocol B were significantly minor ($p < 0.001$) than following protocol A, for each diameter. Therefore, the protocol A appears better than protocol B in presence of bioglass in the scaffold at the first day. This result may support the hypothesis that the BG, if directly in contact with the cells might stimulate their attachment as reported in literature in contrast to the approach with the fibrin gel where the effect is reduced. Especially the ions release could be limited in the gel.

Given the not homogeneous seeding following the protocol C, the experiment was repeated and also CyQuant test at 7 days was carried out to assess the DNA content and calculate cell numbers.

CyQuant data at 7 days of 1.2 mm PLLA-BG are reported as being representative for all scaffolds (Figure 7.18). The results plotted confirm the hypothesis formulated since only in the scaffold with fibrin gel the cell amount did not decrease. This confirms what was reported by Esfahani et al. 2019. The seeding in fibrin gel was individuated how the better seeding technique on scaffold with BG. In this way it can overcome the problem of the high reactivity of BG that can reduce the cell viability.

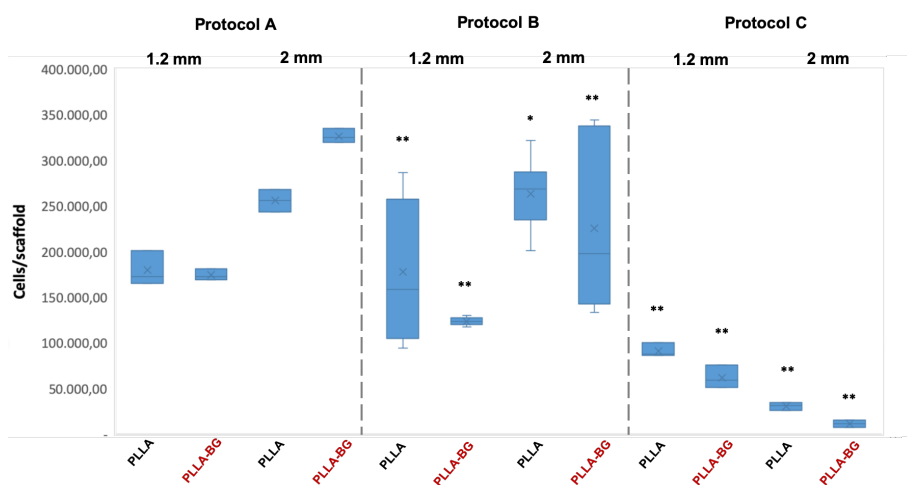


Figure 7.17 Results of the CyQuant proliferation assay in terms of cell numbers present in each scaffold after 1 day of culture. Asterisks denote a significant difference compared with the protocol A, * $P < 0.05$, ** $P < 0.001$.

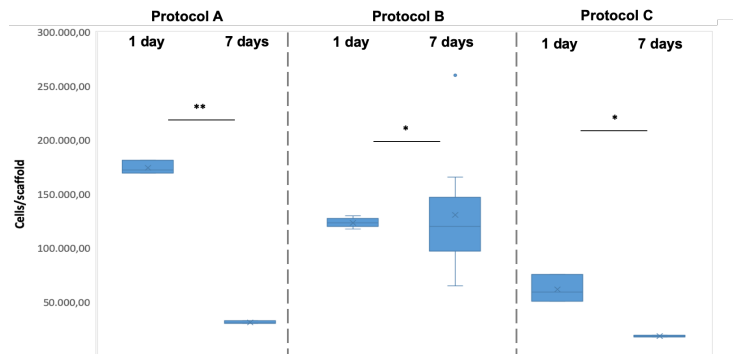


Figure 7.18 Results of the CyQuant proliferation assay in terms of cell numbers present in each scaffold for 1.2 mm PLLA-BG after 1 and 7 day of culture. Asterisks denote a significant difference compared with the protocol A, * $P < 0.05$, ** $P < 0.001$.

In summary, the results showed cell adhesion to the tube wall with all seeding techniques applied even though in protocol A and B cell distribution was more homogenous. Live/dead assay showed that a lot of cells on the tubular scaffolds were viable and metabolically active with orientation towards longitudinal axis. Qualitatively, fibrin gel seeding appeared to have permitted a more effective distribution of cells, according to Esfahani et al. 2019. It appears helpful to achieve an immediate high cell seeding efficiency and to maintain the cells live into scaffolds filled with BG. However, in each test we observed that colonized areas expanded along culture time and the majority of cell survived irrespectively of seeding techniques. Moreover, an essential result is that inside the tube the cells were elongated like typical ligament fibroblasts orientated mostly parallel to the longitudinal tube axis.

7.2 In vitro bioglass cytotoxicity test

Due to their high reactivity once in contact with water-based solutions BGs rapidly exchange ions with the surrounding environment leading in most cases to an undesired increase of the pH making it difficult to perform cell culture studies (Ciraldo et al. 2018). In this paragraph the effect that the release of BG ions could have on different cell lines will be investigated.

7.2.1 Cell culture

The cytotoxicity of the BG 45S5 and BG13-93 powders was assessed using two different cell lines: ACL fibroblasts and L929 cells. The L929 cell line was chosen because of its high proliferation rate in short time. Lapine ACL fibroblast (LKB) cells were cultured in a complete cell culture medium (CCM) as described in the paragraph 7.1.3. Instead, the L929 cell line was culture in different conditions by using a different culture medium, composed of DMEM/Ham's (1:1) FG 4815, 1% Penicillin-Streptomycin, 1% Partricin, 1% ess As, 1% ascorbic acid and 1% Gentamycin.

Cells were seeded into 96-well tissue culture plates at a density of 10,000 cell/well to perform the viability assay. To evaluate the viability by live/dead staining and the morphology by hematoxylin/eosin assay, the cells were seeded on the covered poly-L-lysine glasses with a cell density of 10,000 cell/cm² and allocated into 24 well-plate.

7.2.2 Preparation of CCM with BG dissolution products

According to the protocol proposed by *P.Balasubramanian et al. 2016*, the BG45S5 and BG13-93 powders were sterilized at 160°C for 2 h in a furnace to prepare the medium

containing the ionic dissolution products (IDPs) of BG. Briefly, the experimental set-up for the cell biology study is schematically described below:

- The BG powders was placed in a falcon tube and pre-incubated for 48 h with appropriate medium. 0.5 g of powder were added to 10 ml of CCM to obtained a 5% wt/vol suspension;
- The day after, the cells were seeded both in the 96- well plate, both on cover slips and incubated with CCM for 24 h, to favor the attachment on the surface;
- After 48 h of preconditioning, 10 ml CCM with the IDPs were diluted to form solutions with concentrations of 0.5 and 0.05 wt/vol% of BG in CCM. Solutions at the same concentration where prepared in triplicate (n=3) and for each one the pH was measured by pHmeter;
- The cells were incubated for 24 h with the prepared solutions at three different concentration

In order to carry out viability test and live/dead staining, cells cultured in the CCM were taken as a positive control, while cells cultured in medium added with 10% of Dimethyl sulfoxide (DMSO) were considered as negative control. The test was performed for each concentration in six replicate (n=6).

Cell viability was determined by MTT assay method (3-[4,5-dimefhylfhiazol-2-yl]-2,5-diphenylfetrazolium bromide). To quantify the cells, the medium was removed from the 96-well plate and the cells were incubated with 100 μ L MTT solution in cell culture

medium (1:4 v/v) for 2 h at 37 °C. The values were estimated by spectrophotometric analysis at 490 nm. Cell viability percentage was reported as relative viability compared to cells which were cultured with fresh CCM.

To further confirm the viability of cells cultured with medium with different concentration of BG ions, cells seeded in the glasses were stained with a live\dead and with H&E solution, following the protocols described above.

7.2.3 Results

Before incubating cells with solutions containing IDPs, morphology of L929 and ACL fibroblast were observed under a phase-contrast inverted microscope. Cells exhibited a typical phenotype, L929 cells appeared round or ovoid in shape, while ACL showed a typical fibroblastic morphology Figure 7.19.

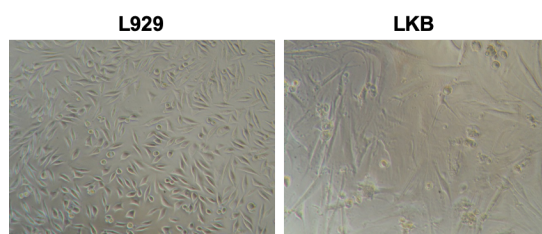


Figure 7.19 Representative phase contrast photomicrographs of L929 and lapine ACL cell cultures, before incubating with the medium containing BG ionic dissolution products.

As can be seen in Figure 7.20, an increase in pH occurring in 48 hours of the immersion was presented for all solutions. The medium pH became alkaline due to ions release of BG45S5 and BG13-93 (Cerruti, Greenspan, and Powers 2005; Vollenweider et al. 2007). At lower concentration (0.05%) the pH variation was not appreciating between two BGs. The solution is so diluted that any effect on the pH was identified. On the contrary the pH value of solutions at 5% concentration was more alkaline than pH of control for both powders. The pH of these solutions was 9.63 ± 0.1 and 9.24 ± 0.01 for the IDPs of BG45S5 and BG13-93, respectively.

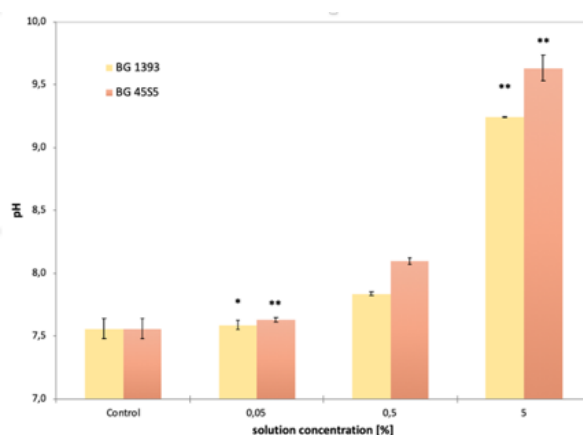


Figure 7.20 Changes in pH caused by ions release of BG45S5 and BG13-93. pH values of solutions at different concentrations. Fresh medium was used as control. The results represent average value of measurements ($n=3$) with error bars giving SD values. Statistical significance levels were set at $p < 0.05$ (), $p < 0.01$ (**)*

Previous studies (Silver, Deas, and Erecin 2001) have demonstrated that the BG releases ions in aqueous solution influencing the cell growth. This finding was confirmed in this study. The Figure 7.21 visualizes the live/dead staining of ACL fibroblasts cultured with solutions of different concentrations. Obviously, at 5% concentration a lot of cells did not survive because of the medium became alkaline. The same behavior was expressed by L929 cells (Figure 7.23). Also hematoxylin/eosin staining supports these data, both for ACL fibroblast (Figure 7.22) and for L929 (Figure 7.24). According to the Normative ISO 10993-5_2009, it can be affirmed that BG45S5 in solution at higher concentration was slight cytotoxic because cell layers were not completely destroyed, but more than 50% growth inhibition was observable.

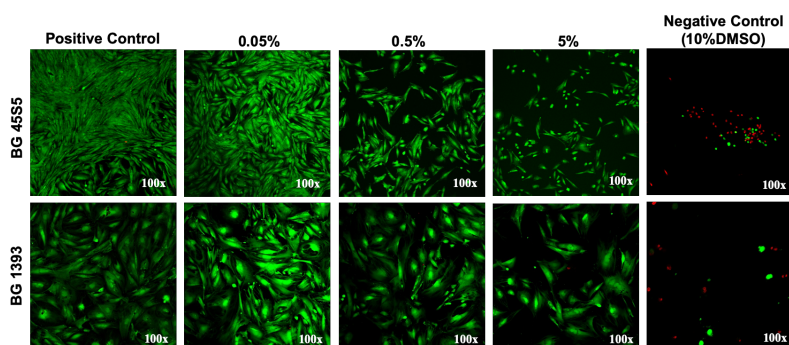


Figure 7.21 Live/dead staining of ACL fibroblast in culture with IDPs of BG45S5 and BG13-93 at different concentration

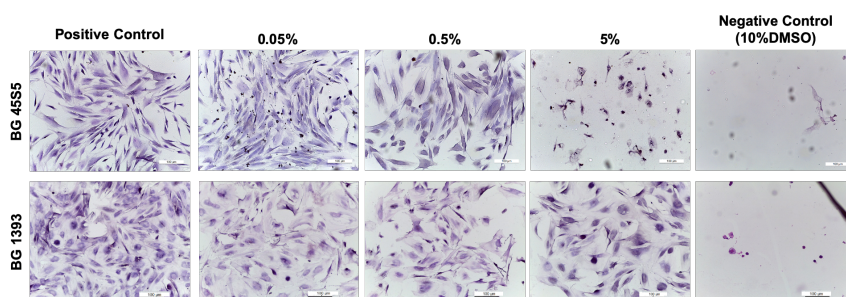


Figure 7.22 Hematoxylin/Eosin staining of ACL fibroblast in culture with IDPs of BG45S5 and BG13-93 at different concentration

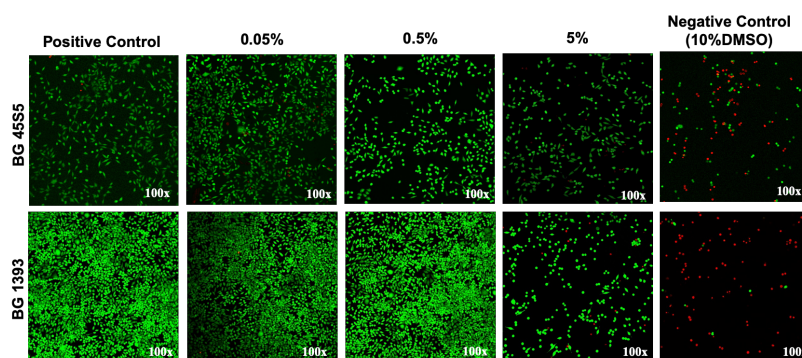


Figure 7.23 Live/dead staining of L929 in culture with IDPs of BG45S5 and BG13-93 at different concentration

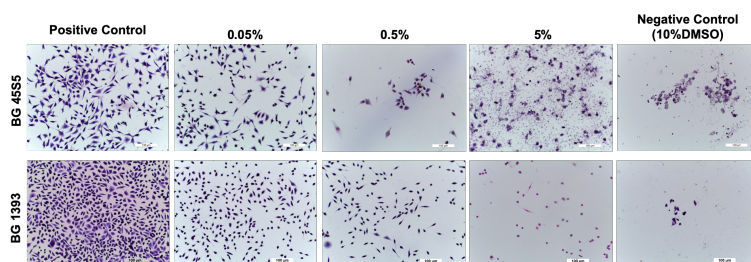


Figure 7.24 Hematoxylin/Eosin staining of L929 in culture with IDPs of BG45S5 and BG1393 at different concentration

Confocal images obtained from cells cultured with BG solution at different concentration were processed with Image J software to assess the percentage of covered area by living cells in respect to total area.

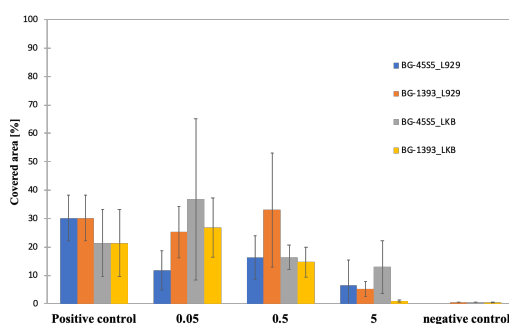


Figure 7.25 the percentage of colonized area of living cells respect the total area of the live/dead picture.

From the graph a higher LKB cell viability than in the control can be appreciated with a solution at 0.05% of IDPs of BG1393. The MTT data (Figure 7.26) revealed that the solutions with 5% of IDPs of BG13-93 reduces significantly the cell viability ($p < 0.01$) of both cell types. Though this glass showed a slight reduction of cell viability, it was not higher than 30% therefore, the BG cannot be considered cytotoxic at a concentration of 5% (Normative ISO 10993-5 2009). Furthermore, it can be seen that LKB cells cultured with solutions of BG45S5 at 0.5% and 0.05% showed the higher cell viability ($p \ll 0.01$). A cell viability higher than the control was also expressed by L929 when cultured with a solution of BG13-93 at 5%. No significant differences in cellular activity of L929 were observed at all concentrations tested in presence of BG 45S5. A significant reduction ($p < 0.01$) of the metabolic activity of LKB was evident at all concentrations for the 13-93 release of products. This result is contrary to a previous study (Qazi et al. 2017) which demonstrated a less pronounced on the viability of hMSCs by 13-93 mediated release of products compared with that of 45S5. A possible explanation for this might depend on the cell type used.

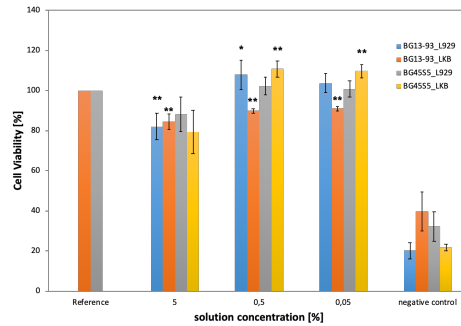


Figure 7.26 Relative viability of LKB, L929 cells IDPs of different BG composition and dilutions. Asterisks denote significant difference compared with the reference, * $p < 0.05$; ** $p < 0.001$

7.3 Bilayer tubular scaffold

The possibility to combine different processing strategies can improve the biomimetic degree of engineered vessels as well as their physical properties. Here, we present new tubular scaffolds with specific surface microstructures for ligaments regeneration. The internal layer of the tubular scaffolds are fabricated by diffusion induced phase separation (DIPS) that permits high control of the pore size, internal diameter and thickness. The external layer was produced by rotationally deposition of electrospun (ES) fibers. Because of the nanofibrous and highly interconnected porous structure of the electrospun layer, likely able to mimic the extracellular matrix of living tissues, the bilayer tubular scaffolds are biomimetic and can help the regeneration of each layer of the tubular structure.

7.3.1 Scaffold preparation

DIPS layer was produced by dissolving PLLA (8 wt%) in neat dioxane at 120 °C. A stainless steel wire ($D = 1.2$ mm) was immersed into the solution, cooled at 40 °C and then pulled out at a constant speed of 16 cm/min to ensure a proper final average membrane thickness of 120 μm . The coated slide was immersed for 10 minutes into a pure water bath to ensure a complete phase separation. The layers were the dried at room temperature overnight.

Electrospun layer was prepared as follow: PLA was dissolved (10 wt%) in TCM/Ac (2:1 volume) by continuous magnetic stirring overnigth at room temperature. A semi-industrial electrospinning equipment (NF-103, MECC CO., LTD., Japan) was used to prepare PLA layer. The polymeric solution was filled in a syringe fitted stainless steel needle. The electrospinning was then performed at flow rate = 1 ml/h and supplied high voltage = 15 kV. The nanofibers obtained were collected on the DIPS-made layers put on a grounded rotary system rotating at 10 rpm for 0.5 hours. The scaffolds were dried for at least 2 days under fume hood in order to remove any residual solvents (Scaffaro, Lopresti, and Botta 2017).

7.3.2 Scaffold characterization

Morphology of the materials was studied by using a Scanning Electron Microscope (SEM).

Tensile mechanical measurements were carried out by using a dynamometer (Instron model 3365) equipped with BioPuls Bath (Norwood, MA) on rectangular shaped specimens (10×90 mm) cut off from films prepared as described above.

7.3.1 Results

Morphological analysis by SEM conducted on DIPS-made tubes revealed that their internal surface (Figure 7.27A) was homogeneously structured with micropores sized around 5 μm and a mean thickness of 120 μm . Micrograph of Electrospun mats in Figure 7.27B showed the typical morphology of an electrospun material with smooth and homogenous fibers in the nearly-nanoscale and randomly oriented.

In Figure 7.27 C, D the cross section of the bilayer tube is reported. It is possible to observe that the outer layer made of ES PLA is characterized by the typical fibrous morphology of electrospun mats with a thickness comparable with that of the DIPS-made tube in the inner layer. In the magnification of Figure 7.27 D the interface between the layers appears well joined without internal cleavages and no matrix discontinuity.

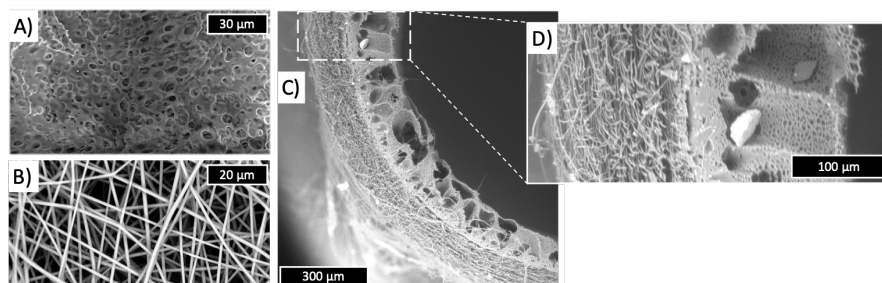


Figure 7.27 SEM micrographs of A) internal layer of DIPS-made tube; B) electrospun mats; (C, D) bilayer tubular scaffold.

Table 7.3 Mechanical properties of tubular scaffold produced via DIPS, electrospun fibers and by-layer scaffold DIPS/ES

	E [MPa]	TS [MPa]	ϵ (%)
DIPS	498.7 ± 18.5	8.12 ± 0.86	30.0 ± 7.1
DIPS/ES	267.2 ± 10.1	4.22 ± 0.51	31.1 ± 8.2
ES	8.64 ± 0.53	2.53 ± 0.15	141.7 ± 13.3

In Table 7.3 the mechanical parameters obtained from stress strain curves are summarized. DIPS tube Young's modulus is more than 50 times higher than that of ES membranes while tensile strength is more than threefold. These results can be ascribed to the high porosity of electrospun membranes that affect their strength. On the other hand, the electrospun membranes showed a much higher elongation at break if compared with

DIPS systems. As expected, the bilayer tubes showed Young's modulus and tensile strength approximately half than that of DIPS tube and the same deformation at break. This result can be simply explained by taking into account that this system can be considered as two in-parallel materials presenting, approximately, the same volume fraction. The low deformation can be ascribed to the impulsive stress that the inner layer, during its break, transfers to the ES layer, thus inducing the fracture of the whole system. Previously biological tests carried out on tubular scaffold produced via DIPS confirm that this structure helps the formation of a homogeneous cells layer similar to ligaments real tissue. On the contrary, how reported in others works (Lopresti et al. 2020; Scaffaro et al. 2017), cells culture on electrospun fiber are very difficult to perform because the cells slither from mesh. So, it is not possible to have the complete support colonization like in other. Morphological and mechanical properties of this new tubular scaffolds, make them highly promising for the construction of biomimetic support for ACL regeneration.

Chapter 8

Conclusion

In the present PhD work, composite porous scaffolds for bone tissue engineering applications were prepared and characterized. Composite materials were prepared combining polymer (PLLA), hydroxyapatite (HA) and bioactive glass (BG) in order to obtain structure with features and biological properties as much as possible comparable to bone or ligament native tissue.

Choosing simply fabrication technique like Thermally Induced Phase Separation (TIPS) and Diffused Induced Phase Separation (DIPS) was fundamental to obtain good results in term of biological response. The scaffold produced were optimized in terms of pore size distribution, mechanical strength and biocompatibility.

In particular, by selecting adequate process parameters, scaffold with a structure highly porous and a good pore interconnection were obtained.

These features were highlighted through Micro-Computed Tomography (μ CT) as non-invasive analysis technique not yet used to characterize polymeric structure produced via TIPS. Here, the main advantage of this technique was exploited. In particular a specific protocol was proposed for polymeric structures characterization with a high complexity level. Additionally, the use of TIPS as a fabrication process for PLLA-HA scaffold allowed to obtain in a simply way a good structure that promote the cell growth into the scaffolds highlighting a good biocompatibility of the support.

The biological *in vitro* test on PLLA-HA scaffold lead to understand that increasing the HA amount above 5% wt slows down osteoblast cell growth, according to literature. But concurrently, HA address the osteoblastic cells toward the maturation when cell confluence is reached. So, it can be confirmed that HA stimulates the cells and get better the properties of pure PLLA because providers osteoinductive properties lack in the polymer.

The opportunity to combine PLLA-BG with different ions concentration was also investigated. Starting from problem reported in literature, in this work was studied the way to integrate the BG into polymeric scaffold produced via TIPS. Until today's this problem remained un-resolved because the support was very brittle.

Here, the better protocol of BG integration into PLLA matrix was identified. It has been found that the BG pre-treatment does not show any effect on the initial BG 13-93 properties but helps the integration process with an increase in mechanical strength of structures.

Another important application reported in some work is the BG ability to stimulate the release of VEGF, so the angiogenic process. This latter is fundamental in the steps of tissue regeneration. A several BG compositions was investigated previously and the borate was identified as that stimulate cellular VEGF expression but at high concentrations shows an opposite effect, reduction of cell viability.

Composite membranes (PLLA-BGs) were successfully prepared via DIPS using BG at different concentration in order to investigate how the composition can influence the bioactivity and the angiogenic potential of scaffold. Study of bioactivity was led in order to investigate the possibility to form an HA layer on the surface. More in detail, all composite showed a good bioactivity after 14 days and the formation of HCA layer was confirmed by SEM XRD and FTIR analysis. Furthermore, from a biological indirect study on the ST-2 cells was observed that BG ionic dissolution products of PLLA-BG13-93B3 stimulate the cells to secrete VEGF. It was important because the scaffold vascularization is fundamental for the tissue regeneration.

Another strategy was proposed about the production of PLLA-BG-HA scaffold. The start point was to be able to take the best properties of each component. The aim was to overcome limitation due to the use of a single materials such as the low bioactivity of PLLA-HA, the brittleness of PLLA-BG. The results showed that the combination of HA and BG 13-93 as fillers produces scaffolds with homogeneous morphological structure and makes them less brittle than scaffolds with BG alone. Limitation due to the bioactivity of HA alone has not yet been overcome.

The approach proposed for ligament regeneration with tubular scaffolds via DIPS has shown good results. In particular the fibrin gel mediated seeding appeared to have permitted a more effective distribution of cells. It allows to achieve an immediate high cell seeding efficiency. However, in each test it was observed that colonized areas expanded along culture time and the majority of cell survived irrespectively of seeding techniques. Moreover, an essential result is that inside the tube the cells were elongated

like typical ligament fibroblasts into the tube. These supports could be a promising approach of ligament TE to support cell alignment especially if integrated in a multilayer scaffold.

In conclusion, different approaches were adopted in order to study which strategies best work on bone and ligament regeneration based on PLLA combined with HA and/or BG scaffold produced via TIPS and DIPS techniques. The results obtained from each test have allow to open up new horizons on the use of composite scaffolds for bone and ligament regeneration. Finally, the activity carried out highlighted how the adoption of biocompatible scaffolds allows interventions aimed at the regeneration of less invasive tissues with a significant reduction of inflammatory phenomena due to incompatibility problems.

Chapter 9

Future work

This PhD thesis focused on some of the most versatile and easy techniques for developing porous composite scaffold highly porous i.e. Thermally Induced Phase separation (TIPS) and Diffused Induced Phase separation (DIPS). The systematic approach herein adopted to correlate structure-processing-properties relationships has led to satisfactory results both in term of structural features both of biocompatibility.

In future, the possibility to realize dynamic culture into bioreactor of scaffolds in PLLA-HA, PLLA-BG13-93 could help the research to better understand the behavior of these structures in a physiological environment. A further investigation related to the mechanism involved in the BG integration into scaffolds produced via TIPS, could lead to find a solution to increase the BG amount to further boost the bioactivity of PLLA-HA-BG scaffolds.

Furthermore, for the composite scaffold produced via DIPS, a direct test could be designed in order to investigate the interaction of cells with the membrane surface. In addition, may be of interest to investigate the effect of increasing of the BG concentration on the osteoinduction and angiogenic potential of composites. Finally, in the realizing of tubular scaffold for ACL ligaments regeneration a good future perspective could be the integration of this small tubes in larger scaffold in order to obtained structure composed of different layers just like the native tissue with its hierarchical organization and more manageable and easily implantable in vivo.

Reference

- Abdollahi, Sara, Alvin Chih, Chien Ma, and Marta Cerruti. 2013. "Surface Transformations of Bioglass 45S5 during Scaffolds Synthesis for Bone Tissue Engineering." *American chemical society* 29: 1466–74.
- Agrawal, Ashwini K., and Rahul Bhalla. 2003. "Advances in the Production of Poly(Lactic Acid) Fibers. A Review." *Journal of Macromolecular Science - Polymer Reviews* 43(4): 479–503.
- Amini, Ami R., Cato T. Laurencin, and Syam P. Nukavarapu. 2012. "Bone Tissue Engineering: Recent Advances and Challenges." *Critical Reviews in Biomedical Engineering* 40(5): 363–408.
- Andrianov, Alexander K., and Robert Langer. 2009. Polyphosphazenes for Biomedical Applications *Polyphosphazenes for Biology and Medicine: Current Status and Future Prospects*.
- Armentano, I. et al. 2010. "Biodegradable Polymer Matrix Nanocomposites for Tissue Engineering: A Review." *Polymer Degradation and Stability* 95(11): 2126–46. <http://dx.doi.org/10.1016/j.polymdegradstab.2010.06.007>.
- Asghari, Fatemeh et al. 2017. "Biodegradable and Biocompatible Polymers for Tissue Engineering Application: A Review." *Artificial Cells, Nanomedicine and Biotechnology* 45(2): 185–92.
- Atesok, Kivanc et al. 2016. "Multilayer Scaffolds in Orthopaedic Tissue Engineering." *Knee Surgery, Sports Traumatology, Arthroscopy* 24(7): 2365–73.
- Azevedo, M C, and R L Reis. 2003. "Development and Properties of Polycaprolactone / Hydroxyapatite Composite Biomaterials." *JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE* 14: 103–7.
- Azis, Yelmida, Novesar Jamarun, Syukri Arief, and Hadi Nur. 2015. "Facile Synthesis of Hydroxyapatite Particles from Cockle Shells (Anadara granosa) by Hydrothermal Method." *Oriental Journal of Chemistry* 31(2): 1099–1105.
- Balasubramanian, P. et al. 2017. "Angiogenic Potential of Boron-Containing Bioactive Glasses: In Vitro Study." *J Mater Sci* 52: 8785–92.
- Barroca, N., A. L. Daniel-Da-Silva, P. M. Vilarinho, and M. H.V. Fernandes. 2010. "Tailoring the Morphology of High Molecular Weight PLLA Scaffolds through Bioglass Addition." *Acta Biomaterialia* 6(9): 3611–20. <http://dx.doi.org/10.1016/j.actbio.2010.03.032>.

- Baudequin, Timothée, and Maryam Tabrizian. 2018. "Multilineage Constructs for Scaffold-Based Tissue Engineering: A Review of Tissue-Specific Challenges." *Advanced Healthcare Materials* 7(3): 1–29.
- Bejarano, Julian, Rainer Detsch, Aldo R Boccaccini, and Humberto Palza. 2016. "PDLLA Scaffolds with Cu- and Zn-Doped Bioactive Glasses Having Multifunctional Properties for Bone Regeneration." *J Biomed Mater Res Parte B*: 746–56.
- Bellucci, Devis et al. 2019. "SBF Assays, Direct and Indirect Cell Culture Tests to Evaluate the Biological Performance of Bioglasses and Bioglass-Based Composites: Three Paradigmatic Cases." *Materials Science and Engineering C* 96(October 2018): 757–64. <https://doi.org/10.1016/j.msec.2018.12.006>.
- Benders, Kim E.M. et al. 2013. "Extracellular Matrix Scaffolds for Cartilage and Bone Regeneration." *Trends in Biotechnology* 31(3): 169–76.
- Bischel, Lauren L. et al. 2016. "Electrospun Gelatin Biopapers as Substrate for in Vitro Bilayer Models of Blood-Brain Barrier Tissue." *Journal of Biomedical Materials Research - Part A* 104(4): 901–9.
- Björkenheim, R. et al. 2017. "Polymer-Coated Bioactive Glass S53P4 Increases VEGF and TNF Expression in an Induced Membrane Model in Vivo." *Journal of Materials Science* 52(15): 9055–65.
- Blaker, Jonny J, Aldo R Boccaccini, and Showan N Nazhat. 2005. "Thermal Characterizations of Silver-Containing Bioactive Glass-Coated Sutures." *Journal of Biomaterials Applications*, 20.
- Boanini, E., M. Gazzano, and A. Bigi. 2010. "Ionic Substitutions in Calcium Phosphates Synthesized at Low Temperature." *Acta Biomaterialia* 6(6): 1882–94. <http://dx.doi.org/10.1016/j.actbio.2009.12.041>.
- Boccaccini, Aldo R et al. 2010. "Polymer / Bioactive Glass Nanocomposites for Biomedical Applications . A Review." *Composites Science and Technology* 13.
- Boerckel, Joel D., Devon E. Mason, Anna M. McDermott, and Eben Alsberg. 2014. "Microcomputed Tomography: Approaches and Applications in Bioengineering." *Stem Cell Research and Therapy* 5: 144.
- Bostancioglu, R. Beklem et al. 2017. "Adhesion Profile and Differentiation Capacity of Human Adipose Tissue Derived Mesenchymal Stem Cells Grown on Metal Ion (Zn, Ag and Cu) Doped Hydroxyapatite Nano-Coated Surfaces." *Colloids and Surfaces B: Biointerfaces* 155: 415–28. <http://dx.doi.org/10.1016/j.colsurfb.2017.04.015>.

- Bouler, J. M., P. Pilet, O. Gauthier, and E. Verron. 2017. "Biphasic Calcium Phosphate Ceramics for Bone Reconstruction: A Review of Biological Response." *Acta Biomaterialia* 53: 1–12. <http://dx.doi.org/10.1016/j.actbio.2017.01.076>.
- Brougham, Claire M. et al. 2017. "Freeze-Drying as a Novel Biofabrication Method for Achieving a Controlled Microarchitecture within Large, Complex Natural Biomaterial Scaffolds." *Advanced Healthcare Materials* 6(21): 1–7.
- Brown, Roger F. et al. 2008. "Growth and Differentiation of Osteoblastic Cells on 13-93 Bioactive Glass Fibers and Scaffolds." *Acta Biomaterialia* 4(2): 387–96.
- Camarero-Espinosa, Sandra, Barbara Rothen-Rutishauser, E. Johan Foster, and Christoph Weder. 2016. "Articular Cartilage: From Formation to Tissue Engineering." *Biomaterials Science* 4(5): 734–67.
- Carfi Pavia, F. V. La Carrubba, S. Piccarolo, and V. Brucato. 2008. "Polymeric Scaffolds Prepared via Thermally Induced Phase Separation: Tuning of Structure and Morphology." *Journal of Biomedical Materials Research - Part A* 86(2): 459–66.
- Carfi Pavia, Francesco et al. 2018. "Preparation , Characterization and in Vitro Test of Composites Poly-Lactic Acid / Hydroxyapatite Scaffolds for Bone Tissue Engineering." *International Journal of Biological Macromolecules* 119: 945–53. <https://doi.org/10.1016/j.ijbiomac.2018.08.007>.
- Carfi Pavia, Francesco, Vincenzo La Carrubba, Giulio Ghersi, and Valerio Brucato. 2012. "Poly-Left-Lactic Acid Tubular Scaffolds via Diffusion Induced Phase Separation : Control of Morphology." *POLYMER ENGINEERING AND SCIENCE*.
- La Carrubba, V., F. Carfi Pavia, and V. Brucato. 2010. "Tubular Scaffold for Vascular Tissue Engineering Application." *International Journal of Material Forming* 3(SUPPL. 1): 567–70.
- Cerruti, Marta, David Greenspan, and Kevin Powers. 2005. "Effect of PH and Ionic Strength on the Reactivity of Bioglass s 45S5." *Biomaterials* 26: 1665–74.
- Chamchoy, Kamonwan et al. 2019. "Application of WST-8 Based Colorimetric NAD(P)H Detection for Quantitative Dehydrogenase Assays." *BMC Biochemistry* 20(1): 1–14.
- Chen, Qizhi Z, Ian D Thompson, and Aldo R Boccaccini. 2006. "45S5 Bioglass s -Derived Glass – Ceramic Scaffolds for Bone Tissue Engineering." 27: 2414–25.
- Chia, Helena N., and Benjamin M. Wu. 2015. "Recent Advances in 3D Printing of Biomaterials." *Journal of*

Biological Engineering 9(1): 1–14.

- Chieng, Buong Woei et al. 2014. “Effects of Graphene Nanoplatelets and Reduced Graphene Oxide on Poly(Lactic Acid) and Plasticized Poly(Lactic Acid): A Comparative Study.” *Polymers* 6(8): 2232–46.
- Cholas, Rahmatullah et al. 2016. “Scaffolds for Bone Regeneration Made of Hydroxyapatite Microspheres in a Collagen Matrix.” *Materials Science & Engineering C* 63: 499–505. <http://dx.doi.org/10.1016/j.msec.2016.03.022>.
- Ciraldo, Francesca E et al. 2018. “Tackling Bioactive Glass Excessive in Vitro Bioreactivity : Preconditioning Approaches for Cell Culture Tests.” *Acta Biomaterialia* 75: 3–10. <https://doi.org/10.1016/j.actbio.2018.05.019>.
- Conoscenti, Gioacchino, Francesco Carfi Pavia, et al. 2017. “In Vitro Degradation and Bioactivity of Composite Poly-L-Lactic (PLLA)/Bioactive Glass (BG) Scaffolds: Comparison of 45S5 and 1393BG Compositions.” *Journal of Materials Science*. <http://link.springer.com/10.1007/s10853-017-1743-9>.
- Conoscenti, Gioacchino, Tobias Schneider, et al. 2017. “PLLA Scaffolds Produced by Thermally Induced Phase Separation (TIPS) Allow Human Chondrocyte Growth and Extracellular Matrix Formation Dependent on Pore Size.” *Materials Science and Engineering C* 80: 449–59.
- Correlo, V. M. et al. 2009. “Melt-Based Compression-Molded Scaffolds from Chitosan-Polyester Blends and Composites: Morphology and Mechanical Properties.” *Journal of Biomedical Materials Research - Part A* 91(2): 489–504.
- Cox, Sophie C. et al. 2015. “3D Printing of Porous Hydroxyapatite Scaffolds Intended for Use in Bone Tissue Engineering Applications.” *Materials Science and Engineering C* 47: 237–47. <http://dx.doi.org/10.1016/j.msec.2014.11.024>.
- Dehghani, Fariba, and Nasim Annabi. 2011. “Engineering Porous Scaffolds Using Gas-Based Techniques.” *Current Opinion in Biotechnology* 22(5): 661–66. <http://dx.doi.org/10.1016/j.copbio.2011.04.005>.
- Dehnavi, Shiva Soltani, Mehdi Mehdikhani, Mohammad Ra, and Shahin Bonakdar. 2018. “Preparation and in Vitro Evaluation of Polycaprolactone / PEG / Bioactive Glass Nanopowders Nanocomposite Membranes for GTR / GBR Applications.” *Materials Science & Engineering C* 90(December 2017): 236–47.
- Deliormanlı, Aylin M, and Mohamed N Rahaman. 2012. “Direct-Write Assembly of Silicate and Borate Bioactive Glass Scaffolds for Bone Repair.” *Journal of the European Ceramic Society* 32(14): 3637–46.

<http://dx.doi.org/10.1016/j.jeurceramsoc.2012.05.005>.

- Diaz, E, I. Sandonis, I. Puerto, and I. Ibanez. 2014. "In Vitro Degradation of PLLA/NHA Composite Scaffolds." *POLYMER ENGINEERING AND SCIENCE*—.
- Dinarvand, Peyman et al. 2011. "New Approach to Bone Tissue Engineering : Simultaneous Application of Hydroxyapatite and Bioactive Glass Coated on a Poly (L -Lactic Acid) Scaffold." *Applied Materials & Interfaces*: 4518–24.
- Domingos, Marco et al. 2017. "Three-Dimensional Printed Bone Scaffolds: The Role of Nano/Micro-Hydroxyapatite Particles on the Adhesion and Differentiation of Human Mesenchymal Stem Cells." *Proceedings of the Institution of Mechanical Engineers, Part H: Journal of Engineering in Medicine* 231(6): 555–64.
- Dong, Chanjuan, and Yonggang Lv. 2016. "Application of Collagen Scaffold in Tissue Engineering: Recent Advances and New Perspectives." *Polymers* 8(2): 1–20.
- Dulnik, J. et al. 2016. "Biodegradation of Bicomponent PCL/Gelatin and PCL/Collagen Nanofibers Electrospun from Alternative Solvent System." *Polymer Degradation and Stability* 130: 10–21.
- Duthon, V B et al. 2006. "Anatomy of the Anterior Cruciate Ligament." *Knee surgery, sports traumatology, arthroscopy* 14(3): 204–13. <http://www.ncbi.nlm.nih.gov/pubmed/16235056>.
- Dziadek, Michal et al. 2015. "Effect of the Preparation Methods on Architecture , Crystallinity , Hydrolytic Degradation , Bioactivity , and Biocompatibility of PCL / Bioglass Composite Scaffolds." *J Biomed Mater Res Parte B*: 1580–93.
- Esfahani, G. Arash et al. 2019. "Hierarchical Microchannel Architecture in Chitosan/Bioactive Glass Scaffolds via Electrophoretic Deposition Positive-Replica." *Journal of Biomedical Materials Research - Part A*: 1455–65.
- Eslami, H, M Solati-Hashjin, M Tahriri, and F Bakhshi. 2010. "Synthesis and Characterization of Nanocrystalline Hydroxyapatite Obtained by the Wet Chemical Technique." *Materials Science- Poland* 28(1): 5–13. <http://www.scopus.com/inward/record.url?eid=2-s2.0-77950939345&partnerID=40&md5=8967f71feef9a3f6aaa2b20335fb9479>.
- Fabbri, Paola et al. 2010. "Highly Porous Polycaprolactone-45S5 Bioglass Ò Scaffolds for Bone Tissue Engineering." *Composites Science and Technology* 70(13): 1869–78. <http://dx.doi.org/10.1016/j.compscitech.2010.05.029>.

- Fan, Hongbin et al. 2008. "In Vivo Study of Anterior Cruciate Ligament Regeneration Using Mesenchymal Stem Cells and Silk Scaffold." *Biomaterials* 29(23): 3324–37.
- Feng, Bei et al. 2015. "Effect of Inhomogeneity of the Electrospun Fibrous Scaffolds of Gelatin/Polycaprolactone Hybrid on Cell Proliferation." *Journal of Biomedical Materials Research - Part A* 103(2): 431–38.
- Font Tellado, Sonia, Elizabeth R. Balmayor, and Martijn Van Griensven. 2015. "Strategies to Engineer Tendon/Ligament-to-Bone Interface: Biomaterials, Cells and Growth Factors." *Advanced Drug Delivery Reviews* 94: 126–40. <http://dx.doi.org/10.1016/j.addr.2015.03.004>.
- Freire-Gormaly, Marina, Jonathan S. Ellis, Aimy Bazylak, and Heather L. MacLean. 2015. "Comparing Thresholding Techniques for Quantifying the Dual Porosity of Indiana Limestone and Pink Dolomite." *Microporous and Mesoporous Materials* 207: 84–89. <http://dx.doi.org/10.1016/j.micromeso.2015.01.002>.
- Frydrych, Martin, and Biqiong Chen. 2013. "Large Three-Dimensional Poly(Glycerol Sebacate)-Based Scaffolds – a Freeze-Drying Preparation Approach." *Journal of Materials Chemistry B*: 6650–61.
- Frydrych, Martin, Sabiniano Román, Sheila Macneil, and Biqiong Chen. 2015. "Biomimetic Poly (Glycerol Sebacate)/ Poly (L -Lactic Acid) Blend Scaffolds for Adipose Tissue Engineering." *Acta Biomaterialia* 18: 40–49. <http://dx.doi.org/10.1016/j.actbio.2015.03.004>.
- Fu, Chuan et al. 2017. "Enhanced Cell Proliferation and Osteogenic Differentiation in Electrospun PLGA/Hydroxyapatite Nanofibre Scaffolds Incorporated with Graphene Oxide." *PLoS ONE* 12(11): 1–20.
- Fu, Qiang et al. 2010. "Silicate , Borosilicate , and Borate Bioactive Glass Scaffolds with Controllable Degradation Rate for Bone Tissue Engineering Applications . II . In Vitro and in Vivo Biological Evaluation." *Wiley periodical*: 172–79.
- Gao, G. et al. 2014. "Bioactive Nanoparticles Stimulate Bone Tissue Formation in Bioprinted Three-Dimensional Scaffold and Human Mesenchymal Stem Cells." *Biotechnology Journal*: 1304–11.
- García-Gareta, Elena, Melanie J. Coathup, and Gordon W. Blunn. 2015. "Osteoinduction of Bone Grafting Materials for Bone Repair and Regeneration." *Bone* 81: 112–21. <http://dx.doi.org/10.1016/j.bone.2015.07.007>.
- Gentile, Piergiorgio et al. 2015. "Process Optimisation to Control the Physico-Chemical Characteristics of Biomimetic Nanoscale Hydroxyapatites Prepared Using Wet Chemical Precipitation." *Materials* 8(5): 1–12.

2297–2310.

- Gerhardt, Lutz-Christian, and Aldo R. Boccaccini. 2010. "Bioactive Glass and Glass-Ceramic Scaffolds for Bone Tissue Engineering." *Materials* 3: 3867–3910. <http://www.mdpi.com/1996-1944/3/7/3867/%5Cnhttp://www.nature.com/doifinder/10.1038/srep04712%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/25152510>.
- Ghassemi, Toktam et al. 2018. "Current Concepts in Scaffolding for Bone Tissue Engineering." *Archives of Bone and Joint Surgery* 6(2): 90–99.
- Gherzi, Giulio et al. 2016. "PLLA Scaffold via TIPS for Bone Tissue Engineering." *Chemical Engineering Transactions* 49: 301–6.
- Ghosh, S, J C Viana, R L Reis, and J F Mano. 2008. "Bi-Layered Constructs Based on Poly (L -Lactic Acid) and Starch for Tissue Engineering of Osteochondral Defects." *Materials Science and Engineering C* 28: 80–86.
- Haafiz, M. K.Mohamad et al. 2013. "Properties of Polylactic Acid Composites Reinforced with Oil Palm Biomass Microcrystalline Cellulose." *Carbohydrate Polymers* 98(1): 139–45. <http://dx.doi.org/10.1016/j.carbpol.2013.05.069>.
- Hahn, Judith et al. 2019. "Viscoelastic Behavior of Embroidered Scaffolds for ACL Tissue Engineering Made of PLA and P(LA-CL) After In Vitro Degradation." *International Journal of Molecular Sciences* 20(18): 4655.
- Haider, Adnan, Kailash Chandra Gupta, and Inn Kyu Kang. 2014. "Morphological Effects of HA on the Cell Compatibility of Electrospun HA/PLGA Composite Nanofiber Scaffolds." *BioMed Research International* 2014.
- Hassan, Mohd Izzat, Tao Sun, and Naznin Sultana. 2014. "Fabrication of Nanohydroxyapatite/Poly(Caprolactone) Composite Microfibers Using Electrospinning Technique for Tissue Engineering Applications." *Journal of Nanomaterials* 2014.
- Haugen, H.J., and S. Bertoldi. 2017. Characterization of Polymeric Biomaterials *Characterization of Morphology—3D and Porous Structure*. Elsevier Ltd. <http://dx.doi.org/10.1016/B978-0-08-100737-2.00002-9>.
- Hench, Larry L. 2006. "The Story of Bioglass." *J Mater Sci: Mater Med* 17: 967–78.

- Herrera-Kao, W. A. et al. 2018. "Thermal Degradation of Poly(Caprolactone), Poly(Lactic Acid), and Poly(Hydroxybutyrate) Studied by TGA/FTIR and Other Analytical Techniques." *Polymer Bulletin* 75(9): 4191–4205.
- Hildebrand, T, and P Ru. 1997. "A New Method for the Model-Independent Assessment of Thickness in Three-Dimensional Images." 185(September 1996): 67–75.
- Hiu-Yan, Yeung et al. 2005. "Novel Approach for Quantification of Porosity for Biomaterial Implants Using Microcomputed Tomography (MCT)." *Wiley InterScience*: 234–42.
- Ho, Tuan Saey, and Dietmar W Hutmacher. 2006. "A Comparison of Micro CT with Other Techniques Used in the Characterization of Scaffolds." *Biomaterials* 27: 1362–76.
- Hoarau-Véchet, Jessica, Arash Rafii, Cyril Touboul, and Jennifer Pasquier. 2018. "Halfway between 2D and Animal Models: Are 3D Cultures the Ideal Tool to Study Cancer-Microenvironment Interactions?" *International Journal of Molecular Sciences* 19(1).
- Hong, Dun et al. 2010. "Morphological and Proteomic Analysis of Early Stage of Osteoblast Differentiation in Osteoblastic Progenitor Cells." *Experimental Cell Research* 316(14): 2291–2300. <http://dx.doi.org/10.1016/j.yexcr.2010.05.011>.
- Hong, Zhongkui et al. 2004. "Grafting Polymerization of L -Lactide on the Surface of Hydroxyapatite Nano-Crystals." *Polymer* 45: 6699–6706.
- Hoppe, Alexander, Nusret S. Güldal, and Aldo R. Boccaccini. 2011. "A Review of the Biological Response to Ionic Dissolution Products from Bioactive Glasses and Glass-Ceramics." *Biomaterials* 32(11): 2757–74. <http://dx.doi.org/10.1016/j.biomaterials.2011.01.004>.
- Hoyer, M. et al. 2014. "In Vitro Characterization of Self-Assembled Anterior Cruciate Ligament Cell Spheroids for Ligament Tissue Engineering." *Histochemistry and Cell Biology* 143(3): 289–300.
- Hua, Fang Jun, Jae Do Nam, and Doo Sung Lee. 2001. "Preparation of a Macroporous Poly(L-Lactide) Scaffold by Liquid-Liquid Phase Separation of a PLLA/1,4-Dioxane/Water Ternary System in the Presence of NaCl." *Macromolecular Rapid Communications* 22(13): 1053–57.
- Huang, Boyang et al. 2018. "Polymer-Ceramic Composite Scaffolds : The Effect of Hydroxyapatite and β -Tri-Calcium Phosphate." *Materials* 11: 129.

- Huang, Jianghong et al. 2013. "Investigation of the In Vitro Degradation of a Novel Polylactide / Nanohydroxyapatite Composite for Artificial Bone." *Journal of Nanomaterials*.
- Inan, T Y. 2017. Recent Developments in Polymer Macro, Micro and Nano Blends *Thermoplastic-Based Nanoblends: Preparation and Characterizations*. Elsevier Ltd. <http://dx.doi.org/10.1016/B978-0-08-100408-1.00002-9>.
- Iqbal, Naseer et al. 2019. "Recent Concepts in Biodegradable Polymers for Tissue Engineering Paradigms: A Critical Review." *International Materials Reviews* 64(2): 91–126. <https://doi.org/10.1080/09506608.2018.1460943>.
- Ishaug, Susan L. et al. 1997. "Bone Formation by Three-Dimensional Stromal Osteoblast Culture in Biodegradable Polymer Scaffolds." *Journal of Biomedical Materials Research* 36(1): 17–28.
- Ji, Chengdong et al. 2012. "Fabrication of Poly- DL-Lactide/Polyethylene Glycol Scaffolds Using the Gas Foaming Technique." *Acta Biomaterialia* 8(2): 570–78. <http://dx.doi.org/10.1016/j.actbio.2011.09.028>.
- Ji, Lijun et al. 2015. "In Vitro Bioactivity and Mechanical Properties of Bioactive Glass Nanoparticles/Polycaprolactone Composites." *Materials Science and Engineering C* 46: 1–9. <http://dx.doi.org/10.1016/j.msec.2014.09.041>.
- Jones, Julian R. 2013. "Review of Bioactive Glass: From Hench to Hybrids." *Acta Biomaterialia* 9: 4457–86. <http://dx.doi.org/10.1016/j.actbio.2012.08.023>.
- Jones, Laurie J. et al. 2001. "Sensitive Determination of Cell Number Using the CyQUANT® Cell Proliferation Assay." *Journal of Immunological Methods* 254(1–2): 85–98.
- Kang, Heemin, Yuze Zeng, and Shyni Varghese. 2018. "Functionally Graded Multilayer Scaffolds for in Vivo Osteochondral Tissue Engineering." *Acta Biomaterialia* 78: 365–77. <https://doi.org/10.1016/j.actbio.2018.07.039>.
- Khajavi, Ramin, Mina Abbasipour, and Abbas Bahador. 2016. "Electrospun Biodegradable Nanofibers Scaffolds for Bone Tissue Engineering." *Journal of Applied Polymer Science* 133(3).
- Kim, J.F. et al. 2017. 1 Comprehensive Membrane Science and Engineering 1.15 *Effect of Solvents on Membrane Fabrication via Thermally Induced Phase Separation (TIPS): Thermodynamic and Kinetic Perspectives*. Elsevier Ltd. <http://dx.doi.org/10.1016/B978-0-12-409547-2.12690-3>.
- Kim, Jo-eun, Won-jin Yi, Min-suk Heo, and Sam-sun Lee. 2015. "Three-Dimensional Evaluation of Human

- Jaw Bone Microarchitecture : Correlation between the Microarchitectural Parameters of Cone Beam Computed Tomography and Micro-Computer Tomography.” *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology* 120(6): 762–70. <http://dx.doi.org/10.1016/j.oooo.2015.08.022>.
- Kim, Tae Hoon et al. 2014. “In Vitro and in Vivo Evaluation of Bone Formation Using Solid Freeform Fabrication-Based Bone Morphogenic Protein-2 Releasing PCL/PLGA Scaffolds.” *Biomedical Materials (Bristol)* 9(2).
- Kneser, U. et al. 2002. “Tissue Engineering of Bone.” *Minimally Invasive Therapy & Allied Technologies* 11: 107–16.
- Kokubo, Tadashi, and Hiroaki Takadama. 2006. “How Useful Is SBF in Predicting in Vivo Bone Bioactivity ?” *Biomaterials* 27: 2907–15.
- Komur, B. et al. 2017. “Characterization of Cu/Ag/Eu/Hydroxyapatite Composites Produced by Wet Chemical Precipitation.” *Acta Physica Polonica A* 131(3): 392–96.
- Krishnan, Vidya, and T. Lakshmi. 2013. “Bioglass: A Novel Biocompatible Innovation.” *Journal of Advanced Pharmaceutical Technology and Research* 4(2): 78–83.
- Langenbach, Fabian, and Jörg Handschel. 2013. “Effects of Dexamethasone, Ascorbic Acid and β -Glycerophosphate on the Osteogenic Differentiation of Stem Cells in Vitro.” *Stem Cell Research and Therapy* 4(5): 1. Stem Cell Research & Therapy.
- Li, Wei et al. 2015. “Multifunctional Chitosan-45S5 Bioactive Glass-Poly (3- Hydroxybutyrate- Co -3- Hydroxyvalerate) Microsphere Composite Membranes for Guided Tissue / Bone Regeneration.” *ACS Appl. Mater. Interfaces* 7: 20845–20845.
- Liu, Mei et al. 2017. “Injectable Hydrogels for Cartilage and Bone Tissue Engineering.” *Bone Research* 5(November 2016).
- Liu, Xiaohoua, and Peter X Ma. 2004. “Polymeric Scaffolds for Bone Tissue Engineering.” *Annals of Biomedical Engineering* 32(3): 477–86.
- Lombardo, Maria Elena et al. 2019. “PLLA Scaffolds with Controlled Architecture as Potential Microenvironment for in Vitro Tumor Model.” *Tissue and Cell* 58(January): 33–41. <https://doi.org/10.1016/j.tice.2019.04.004>.

- Lopresti, Francesco et al. 2020. "Effect of Hydroxyapatite Concentration and Size on Morpho-Mechanical Properties of PLA-Based Randomly Oriented and Aligned Electrospun Nanofibrous Mats." *Journal of the Mechanical Behavior of Biomedical Materials* 101(September 2019): 103449. <https://doi.org/10.1016/j.jmbbm.2019.103449>.
- Lu, Helen H. et al. 2005. "Anterior Cruciate Ligament Regeneration Using Braided Biodegradable Scaffolds: In Vitro Optimization Studies." *Biomaterials* 26(23): 4805–16.
- Lv, Xiang Guo et al. 2016. "Comparative Study of Different Seeding Methods Based on a Multilayer SIS Scaffold: Which Is the Optimal Procedure for Urethral Tissue Engineering?" *Journal of Biomedical Materials Research - Part B Applied Biomaterials* 104(6): 1098–1108.
- Macon, Anthony L B et al. 2015. "A Unified in Vitro Evaluation for Apatite-Forming Ability of Bioactive Glasses and Their Variants." *J Mater Sci: Mater Med* 26: 115.
- Mannella, Gianluca A. et al. 2014. "Evidence of Mechanisms Occurring in Thermally Induced Phase Separation of Polymeric Systems." *Journal of Polymer Science, Part B: Polymer Physics* 52(14): 979–83.
- Mao, Daoyong et al. 2018. "Porous Stable Poly (Lactic Acid)/ Ethyl Cellulose / Hydroxyapatite Composite Scaffolds Prepared by a Combined Method for Bone Regeneration." *Carbohydrate Polymers* 180(July 2017): 104–11. <http://dx.doi.org/10.1016/j.carbpol.2017.10.031>.
- Marrella, Alessandra, Maurizio Aiello, Rodolfo Quarto, and Silvia Scaglione. 2016. "Chemical and Morphological Gradient Scaffolds to Mimic Hierarchically Complex Tissues: From Theoretical Modeling to Their Fabrication." *Biotechnology and Bioengineering* 113(10): 2286–97.
- Mathieu, Laurence Marcelle et al. 2006. "Architecture and Properties of Anisotropic Polymer Composite Scaffolds for Bone Tissue Engineering." *Biomaterials* 27(6): 905–16.
- Meyer, Ulrich, and Hans Peter Wiesmann. 2006. Bone and Cartilage engineering *Bone and Cartilage*.
- Mol, Anita et al. 2005. "Fibrin as a Cell Carrier in Cardiovascular Tissue Engineering Applications." *Biomaterials* 26(16): 3113–21.
- Molladavoodi, Sara, Maud Gorbet, John Medley, and Hyock Ju Kwon. 2012. "Investigation of Microstructure, Mechanical Properties and Cellular Viability of Poly(L-Lactic Acid) Tissue Engineering Scaffolds Prepared by Different Thermally Induced Phase Separation Protocols." *Journal of the Mechanical Behavior of Biomedical Materials* 17: 186–97. <http://dx.doi.org/10.1016/j.jmbbm.2012.08.021>.

- . 2013. “Investigation of Microstructure, Mechanical Properties and Cellular Viability of Poly(L-Lactic Acid) Tissue Engineering Scaffolds Prepared by Different Thermally Induced Phase Separation Protocols.” *Journal of the Mechanical Behavior of Biomedical Materials* 17: 186–97. <http://dx.doi.org/10.1016/j.jmbbm.2012.08.021>.
- Montesanto, Salvatore, Gianluca A Mannella, Francesco Carf, and Vincenzo La Carrubba. 2015. “Coagulation Bath Composition and Desiccation Environment as Tuning Parameters to Prepare Skinless Membranes via Diffusion Induced Phase Separation.” *Journal of Applied Polymer Science*: 1–10.
- Moore, Michael J et al. 2004. “Quantitative Analysis of Interconnectivity of Porous Biodegradable Scaffolds with Micro-Computed Tomography.” *Wiley InterScience*.
- Morent, Rino et al. 2011. “Plasma Surface Modification of Biodegradable Polymers: A Review.” *Plasma Processes and Polymers* 8(3): 171–90.
- Morris, David E., Melissa L. Mather, and John A Crowe. 2009. “Biomaterials Generation and Simulated Imaging of Pseudo-Scaffolds to Aid Characterisation by X-Ray Micro CT.” *Biomaterials* 30(25): 4233–46. <http://dx.doi.org/10.1016/j.biomaterials.2009.04.035>.
- Motealleh, Azadeh et al. 2017. “Case Study: Reinforcement of 45S5 Bioglass Robocast Scaffolds by HA/PCL Nanocomposite Coatings.” *Journal of the Mechanical Behavior of Biomedical Materials* 75(April): 114–18.
- Nasajpour, Amir et al. 2018. “A Multifunctional Polymeric Periodontal Membrane with Osteogenic and Antibacterial Characteristics.” *Adv. Funct. Mater* 1703437: 1–8.
- Nau, Thomas, and Andreas Teuschl. 2015. “Regeneration of the Anterior Cruciate Ligament : Current Strategies in Tissue Engineering.” *World Journal of Orthopedics* 6(1): 127–36.
- Nejati, E., V. Firouzdar, M. B. Eslaminejad, and F. Bagheri. 2009. “Needle-like Nano Hydroxyapatite/Poly(l-Lactide Acid) Composite Scaffold for Bone Tissue Engineering Application.” *Materials Science and Engineering C* 29(3): 942–49. <http://dx.doi.org/10.1016/j.msec.2008.07.038>.
- Neto, A. I. et al. 2015. “A Novel Hanging Spherical Drop System for the Generation of Cellular Spheroids and High Throughput Combinatorial Drug Screening.” *Biomaterials Science* 3(4): 581–85.
- Nikpour, Parisa, Hamed Salimi-kenari, Farahnaz Fahimipour, and Sayed Mahmood. 2018. “Dextran Hydrogels Incorporated with Bioactive Glass-Ceramic : Nanocomposite Scaffolds for Bone Tissue Engineering.” *Carbohydrate Polymers* 190(March): 281–94. <https://doi.org/10.1016/j.carbpol.2018.02.083>.

- Ning, Jia et al. 2007. "Synthesis and in Vitro Bioactivity of a Borate-Based Bioglass." *Materials Letters* 61: 5223–26.
- Nokhasteh, Samira et al. 2018. "Effect of Bioactive Glass Nanoparticles on Biological Properties of PLGA/Collagen Scaffold." *Progress in Biomaterials* 7(2): 111–19. <https://doi.org/10.1007/s40204-018-0089-y>.
- Noocaid, Patcharakamon, Vehid Salih, Justus P. Beier, and Aldo R. Boccaccini. 2012. "Osteochondral Tissue Engineering: Scaffolds, Stem Cells and Applications." *Journal of Cellular and Molecular Medicine* 16(10): 2247–70.
- Normative ISO 10993-5. 2009. *International Standard ISO 10993-5 Biological Evaluation of Medical Devices — Tests for in Vitro Cytotoxicity*.
- Ohgushi, Hajime, and Arnold I. Caplan. 1999. "Stem Cell Technology and Bioceramics: From Cell to Gene Engineering." *Journal of Biomedical Materials Research* 48(6): 913–27.
- Otsuki, Bungo et al. 2006. "Pore Throat Size and Connectivity Determine Bone and Tissue Ingrowth into Porous Implants: Three-Dimensional Micro-CT Based Structural Analyses of Porous Bioactive Titanium Implants." *Biomaterials* 27: 5892–5900.
- P.Balasubramanian et al. 2016. "Ion Release , Hydroxyapatite Conversion , and Cytotoxicity of Boron-Containing Bioactive Glass Scaffolds." *International Journal of Applied Glass Science* 7: 206–15.
- Pajoumshariati, Seyedramin, Hadi Shirali, Seyedeh Kimia Yavari, and Sogol Naghavi. 2018. "GBR Membrane of Novel Poly Periodontal Application." *Scientific Reports* 9: 1–16. <http://dx.doi.org/10.1038/s41598-018-25952-1>.
- Pav, Carfi, Francesco et al. 2019. "A 3D-scaffold of PLLA Induces the Morphological Differentiation and Migration of Primary Astrocytes and Promotes the Production of Extracellular Vesicles." *Molecular Medicine Reports* 20: 1288–96.
- Peng, Fei, Xiaohua Yu, and Mei Wei. 2011. "In Vitro Cell Performance on Hydroxyapatite Particles/Poly(L-Lactic Acid) Nanofibrous Scaffolds with an Excellent Particle along Nanofiber Orientation." *Acta Biomaterialia* 7(6): 2585–92. <http://dx.doi.org/10.1016/j.actbio.2011.02.021>.
- Petrie Aronin, C. E. et al. 2008. "Osteogenic Differentiation of Dura Mater Stem Cells Cultured in Vitro on Three-Dimensional Porous Scaffolds of Poly(ε-Caprolactone) Fabricated via Co-Extrusion and Gas Foaming." *Acta Biomaterialia* 4(5): 1187–97.

- Petrigliano, Frank A., David R. McAllister, and Benjamin M. Wu. 2006. "Tissue Engineering for Anterior Cruciate Ligament Reconstruction: A Review of Current Strategies." *Arthroscopy - Journal of Arthroscopic and Related Surgery* 22(4): 441–51.
- Pitaluga, Lucas Hidalgo et al. 2018. "Electrospun F18 Bioactive Glass/PCL—Poly (ϵ -Caprolactone)—Membrane for Guided Tissue Regeneration." *Materials* 11: 400.
- Pradhan, S. et al. 2017. Characterization of Polymeric Biomaterials *NMR, FT-IR and Raman Characterization of Biomaterials*. Elsevier Ltd. <http://dx.doi.org/10.1016/B978-0-08-100737-2.00007-8>.
- Qazi, Taimoor H et al. 2017. "Comparison of the Effects of 45S5 and 1393 Bioactive Glass Microparticles on HMSC Behavior." *biomedical materials research part A*: 2772–82.
- Quarles, L. Darryl et al. 1992. "Distinct Proliferative and Differentiated Stages of Murine MC3T3-E1 Cells in Culture: An in Vitro Model of Osteoblast Development." *Journal of Bone and Mineral Research* 7(6): 683–92.
- Radha, G., S. Balakumar, Venkatesan Balaji, and Vellaichamy Elangovan. 2017. "A Novel Nano-Hydroxyapatite – PMMA Hybrid Scaffolds Adopted by Conjugated Thermal Induced Phase Separation (TIPS) and Wet-Chemical Approach: Analysis of Its Mechanical and Biological Properties." *Materials Science and Engineering: C* 73: 164–72. <http://linkinghub.elsevier.com/retrieve/pii/S0928493116312796>.
- Radin, S. et al. 2005. "Osteogenic Effects of Bioactive Glass on Bone Marrow Stromal Cells." *Journal of Biomedical Materials Research - Part A* 73(1): 21–29.
- Ramesh, Niranjana, Stephen C. Moratti, and George J. Dias. 2017. "Hydroxyapatite–Polymer Biocomposites for Bone Regeneration: A Review of Current Trends Niranjana." *J Biomed Mater Res Part B* 106B: 2046–2057.
- Ratheesh, Greeshma et al. 2017. "3D Fabrication of Polymeric Scaffolds for Regenerative Therapy." *ACS Biomaterials Science and Engineering* 3(7): 1175–94.
- Renghini, Chiara et al. 2009. "Micro-CT Studies on 3-D Bioactive Glass-Ceramic Scaffolds for Bone Regeneration." *Acta Biomaterialia* 5(4): 1328–37.
- Rezayan, Ali Hossein et al. 2017. "Synthesis and Characterization of Biodegradable Semi-Interpenetrating Polymer Networks Based on Star-Shaped Copolymers of ϵ -Caprolactone and Lactide." *Iranian Journal of Pharmaceutical Research* 16(1): 63–73.

- Rezwan, K, Q Z Chen, J J Blaker, and Aldo R. Boccaccini. 2006. "Biodegradable and Bioactive Porous Polymer / Inorganic Composite Scaffolds for Bone Tissue Engineering." *Biomaterials* 27: 3413–31.
- Rigogliuso, Salvatrice et al. 2014. "PLLA/Fibrin Tubular Scaffold: A New Way for Reliable Endothelial Cell Seeding." *Conference Papers in Science* 2014: 1–5.
- Rogina, Anamarija et al. 2016. "Macroporous Poly(Lactic Acid) Construct Supporting the Osteoinductive Porous Chitosan-Based Hydrogel for Bone Tissue Engineering." *Polymer* 98: 172–81.
- Roohani-esfahani, S I et al. 2011. "Acta Biomaterialia Effects of Bioactive Glass Nanoparticles on the Mechanical and Biological Behavior of Composite Coated Scaffolds." *Acta Biomaterialia* 7: 1307–18.
- Roseti, Livia et al. 2017. "Scaffolds for Bone Tissue Engineering: State of the Art and New Perspectives." *Materials Science and Engineering C* 78: 1246–62. <http://dx.doi.org/10.1016/j.msec.2017.05.017>.
- Ryszkowska, Joanna L., Monika Auguścik, Ann Sheikh, and Aldo R. Boccaccini. 2010. "Biodegradable Polyurethane Composite Scaffolds Containing Bioglass® for Bone Tissue Engineering." *Composites Science and Technology* 70(13): 1894–1908.
- Sa, Min Woo, and Jong Young Kim. 2013. "Effect of Various Blending Ratios on the Cell Characteristics of PCL and PLGA Scaffolds Fabricated by Polymer Deposition System." *International Journal of Precision Engineering and Manufacturing* 14(4): 649–55.
- Sadat-Shojai, Mehdi, Mohammad Taghi Khorasani, Ehsan Dinpanah-Khoshdargi, and Ahmad Jamshidi. 2013. "Synthesis Methods for Nanosized Hydroxyapatite with Diverse Structures." *Acta Biomaterialia* 9(8): 7591–7621. <http://dx.doi.org/10.1016/j.actbio.2013.04.012>.
- Saghiri, Mohammad-ali, Armen Asatourian, Franklin Garcia-godoy, and Nader Sheibani. 2016. "The Role of Angiogenesis in Implant Dentistry Part II: The Effect of Bone-Grafting and Barrier Membrane Materials on Angiogenesis." *Journal section: Oral Surgery* 21(4).
- Salerno, A., S. Iannace, and P. A. Netti. 2012. "Graded Biomimetic Osteochondral Scaffold Prepared via CO₂ Foaming and Micronized NaCl Leaching." *Materials Letters* 82: 137–40. <http://dx.doi.org/10.1016/j.matlet.2012.05.072>.
- Salvo, L et al. 2003. "X-Ray Micro-Tomography an Attractive Characterisation Technique in Materials Science." *Nuclear Instruments and Methods in Physics Research B* 200: 273–86.
- Sampath, Udeni Gunathilake T.M. et al. 2016. "Fabrication of Porous Materials from Natural/Synthetic

- Biopolymers and Their Composites.” *Materials* 9(12): 1–32.
- Santos, M.L. et al. 2017. “Biomaterials as Tendon and Ligament Substitutes: Current Developments.”
- Sanyanga, M.L. et al. 2016. “Problem Analysis and Development Strategy of Shrimp Culture in Tanah Laut Regency, South Kalimantan Province, Indonesia.” *Carbohydrate Polymers* 146: 36–45. <http://dx.doi.org/10.1016/j.carbpol.2016.03.051>.
- Sarker, Bapi, Jasmin Hum, Showan N. Nazhat, and Aldo R. Boccaccini. 2015. “Combining Collagen and Bioactive Glasses for Bone Tissue Engineering: A Review.” *Advanced Healthcare Materials* 4(2): 176–94.
- Scaffaro, Roberto et al. 2017. “Electrospun PCL/GO-g-PEG Structures: Processing-Morphology-Properties Relationships.” *Composites Part A: Applied Science and Manufacturing* 92: 97–107. <http://dx.doi.org/10.1016/j.compositesa.2016.11.005>.
- Scaffaro, Roberto, Francesco Lopresti, and Luigi Botta. 2017. “Preparation, Characterization and Hydrolytic Degradation of PLA/PCL Co-Mingled Nanofibrous Mats Prepared via Dual-Jet Electrospinning.” *European Polymer Journal* 96: 266–77. <http://dx.doi.org/10.1016/j.eurpolymj.2017.09.016>.
- Schuhladen, Katharina, Xiaoju Wang, Leena Hupa, and Aldo R. Boccaccini. 2018. “Dissolution of Borate and Borosilicate Bioactive Glasses and the Influence of Ion (Zn, Cu) Doping in Different Solutions.” *Journal of Non-Crystalline Solids* 502(August): 22–34.
- Seong, Yun Jeong et al. 2017. “Calcium Phosphate–Collagen Scaffold with Aligned Pore Channels for Enhanced Osteochondral Regeneration.” *Advanced Healthcare Materials* 6(24): 1–11.
- Shavandi, Amin et al. 2015. “Development and Characterization of Hydroxyapatite/β-TCP/Chitosan Composites for Tissue Engineering Applications.” *Materials Science and Engineering C* 56: 481–93. <http://dx.doi.org/10.1016/j.msec.2015.07.004>.
- Shearer, Tom et al. 2016. “Three-Dimensional Visualisation of Soft Biological Structures by X-Ray Computed Micro-Tomography.” *Journal of Cell Science* 129(13): 2483–92. <http://jcs.biologists.org/lookup/doi/10.1242/jcs.179077>.
- Shu, R., R. McMullen, M. J. Baumann, and L. R. McCabe. 2003. “Hydroxyapatite Accelerates Differentiation and Suppresses Growth of MC3T3-E1 Osteoblasts.” *Journal of Biomedical Materials Research - Part A* 67(4): 1196–1204.

- Sikavitsas, Vassilios I., Johnna S. Temenoff, and Antonios G. Mikos. 2001. "Biomaterials and Bone Mechanotransduction." *Biomaterials* 22(19): 2581–93.
- Silver, Ian A, Judith Deas, and Maria Erecin. 2001. "Interactions of Bioactive Glasses with Osteoblasts in Vitro : Effects of 45S5 Bioglass , and 58S and 77S Bioactive Glasses on Metabolism , Intracellular Ion Concentrations and Cell Viability." *Biomaterials* 22: 175–85.
- Singh, Neeraj. 2018. "International Epidemiology of Anterior Cruciate Ligament Injuries." *Orthopedic Research*: 3–5. <https://www.researchgate.net/publication/323989348>.
- Smith, E.R., and M. Wojciechowski. 1937. "Boiling-Point-Composition Digram of the System Dioxane-Water." *Journal of Research of the National Bureau of Standards* 18(4): 461.
- Smith, Ian O., Laura R. McCabe, and Melissa J. Baumann. 2006. "MC3T3-E1 Osteoblast Attachment and Proliferation on Porous Hydroxyapatite Scaffolds Fabricated with Nanophase Powder." *International Journal of Nanomedicine* 1(2): 189–94.
- Sola, Antonella et al. 2019. "Development of Solvent-Casting Particulate Leaching (SCPL) Polymer Scaffolds as Improved Three-Dimensional Supports to Mimic the Bone Marrow Niche." *Materials Science and Engineering C* 96(October 2018): 153–65. <https://doi.org/10.1016/j.msec.2018.10.086>.
- Sommerfeldt, D., and C. Rubin. 2001. "Biology of Bone and How It Orchestrates the Form and Function of the Skeleton." *European Spine Journal* 10: 86–95.
- Stekelenburg, Maria, Marcel C.M. Rutten, Luc H.E.H. Snoeckx, and Frank P.T. Baaijens. 2009. "Dynamic Straining Combined with Fibrin Gel Cell Seeding Improves Strength of Tissue-Engineered Small-Diameter Vascular Grafts." *Tissue Engineering - Part A* 15(5): 1081–89.
- Stoddart, M.J. 2011. "Cell Viability Assay." In *Journal of Chemical Information and Modeling*, , 1–253.
- Subia, B, J Kundu, and S.C. Kundu. 2010. Tissue engineering, InTech *Biomaterials Scaffold Fabrication Techniques for Potential Tissue Engineering Applications*. <https://www.intechopen.com/books/advanced-biometric-technologies/liveness-detection-in-biometrics>.
- Sunandhakumari, Vishnu Jayakumar, Arun Kumar Vidhyadharan, and Aneesh Alim. 2018. "Fabrication and In Vitro Characterization of Bioactive Glass / Nano Hydroxyapatite Reinforced Electrospun Poly (ε - Caprolactone) Composite Membranes for Guided Tissue Regeneration." *bioengineering* 5: 2–20.
- Suntornnond, Ratima, Jia An, Wai Yee Yeong, and Chee Kai Chua. 2015. "Biodegradable Polymeric Films and

- Membranes Processing and Forming for Tissue Engineering.” *Macromolecular Materials and Engineering* 300(9): 858–77.
- Takamuku, Toshiyuki et al. 2001. “NaCl-Induced Phase Separation of 1,4-Dioxane-Water Mixtures Studied by Large-Angle X-Ray Scattering and Small-Angle Neutron Scattering Techniques.” *Journal of Physical Chemistry B* 105(41): 10101–10.
- Tan, Huaping, Constance R. Chu, Karin A. Payne, and Kacey G. Marra. 2009. “Injectable in Situ Forming Biodegradable Chitosan-Hyaluronic Acid Based Hydrogels for Cartilage Tissue Engineering.” *Biomaterials* 30(13): 2499–2506. <http://dx.doi.org/10.1016/j.biomaterials.2008.12.080>.
- Theodoridis, Konstantinos et al. 2019. “Assessment of Cartilage Regeneration on 3D Collagen-Polycaprolactone Scaffolds: Evaluation of Growth Media in Static and in Perfusion Bioreactor Dynamic Culture.” *Colloids and Surfaces B: Biointerfaces* 183(July): 110403. <https://doi.org/10.1016/j.colsurfb.2019.110403>.
- Tillman, Bryan W. et al. 2009. “The in Vivo Stability of Electrospun Polycaprolactone-Collagen Scaffolds in Vascular Reconstruction.” *Biomaterials* 30(4): 583–88. <http://dx.doi.org/10.1016/j.biomaterials.2008.10.006>.
- Tuan, Ho Saey, and Dietmar W Hutmacher. 2005. “Application of Micro CT and Computation Modeling in Bone Tissue Engineering.” 37: 1151–61.
- Türk, S et al. 2018. “Materials Science & Engineering C 3D Porous Collagen / Functionalized Multiwalled Carbon Nanotube / Chitosan / Hydroxyapatite Composite Scaffolds for Bone Tissue Engineering.” *Materials Science & Engineering C* 92(January): 757–68.
- Ureña, J. et al. 2018. “Cellular Behaviour of Bone Marrow Stromal Cells on Modified Ti-Nb Surfaces.” *Materials and Design* 140: 452–59. <https://doi.org/10.1016/j.matdes.2017.12.006>.
- Vitrano, Ilenia et al. 2018. “Evaluation of Hydroxyapatite Distribution in a Poly-L-Lactic Acid (PLLA) Scaffolds via Micro Computed Tomography (MCT).” *Chemical Engineering Transactions* 64: 193–98.
- Vollenweider, Meret et al. 2007. “Remineralization of Human Dentin Using Ultrafine Bioactive Glass Particles.” *Acta Biomaterialia* 3: 936–43.
- Wang, Chen Chie et al. 2011. “A Highly Organized Three-Dimensional Alginate Scaffold for Cartilage Tissue Engineering Prepared by Microfluidic Technology.” *Biomaterials* 32(29): 7118–26. <http://dx.doi.org/10.1016/j.biomaterials.2011.06.018>.

- Wang, Jiaolong et al. 2016. "Biodegradable Polymer Membranes Applied in Guided Bone / Tissue Regeneration : A Review." *Polymers*: 1–20.
- Wang, Wenhao, and Kelvin W K Yeung. 2017. "Bone Graft and Biomaterials Substitute for Bone Defect Repair: A Review." *Bioactive Materials* 2(4): 224–47. <https://doi.org/10.1016/j.bioactmat.2017.05.007>.
- Wetzel, Roland, and Delia S. Brauer. 2019. "Apatite Formation of Substituted Bioglass 45S5: SBF vs. Tris." *Materials Letters*: 126760. <https://doi.org/10.1016/j.matlet.2019.126760>.
- Wilda, Helen, and Julie E Gough Æ. 2006. "In Vitro Studies of Annulus Fibrosus Disc Cell Attachment , Differentiation and Matrix Production on PDLLA / 45S5 Bioglass s Composite Films." *Biomaterials* 27: 5220–29.
- Williams, David F. 2008. "On the Mechanisms of Biocompatibility." *Biomaterials* 29(20): 2941–53.
- De Witte, Tinke-Marie, Lidy E Fratila-Apachitei, Amir A Zadpoor, and Nicholas A Peppas. 2018. "Bone Tissue Engineering via Growth Factor Delivery: From Scaffolds to Complex Matrices." *Regenerative Biomaterials* 5(4): 197–211.
- Xia, Lunguo et al. 2019. "Stimulatory Effects of Boron Containing Bioactive Glass on Osteogenesis and Angiogenesis of Polycaprolactone : In Vitro Study." *BioMed Research International*: 12.
- Xue, Weichang, B. Vamsi Krishna, Amit Bandyopadhyay, and Susmita Bose. 2007. "Processing and Biocompatibility Evaluation of Laser Processed Porous Titanium." *Acta Biomaterialia* 3(6): 1007–18.
- Yan, Xiang-Zhen et al. 2014. "Effects of Continuous Passaging on Mineralization of MC3T3-E1 with Improved Osteogenic Culture Protocol." *Tissue Engineering, Part C* 20.
- Yang, Bao-hua, Ai-xiang Wu, Xiu-xiu Miao, and Jin-zhi Liu. 2014. "3D Characterization and Analysis of Pore Structure of Packed Ore Particle Beds Based on Computed Tomography Images." *Trans. Nonferrous Met. Soc. China* 24: 833–38.
- Yeatts, Andrew B, and John P Fisher. 2011. "Bone Tissue Engineering Bioreactors : Dynamic Culture and the Influence of Shear Stress." *Bone* 48(2): 171–81. <http://dx.doi.org/10.1016/j.bone.2010.09.138>.
- Yoshikawa, Hideki, Noriyuki Tamai, Tsuyoshi Murase, and Akira Myoui. 2009. "Interconnected Porous Hydroxyapatite Ceramics for Bone Tissue Engineering." *Journal of the Royal Society Interface* 6(SUPPL. 3): S341–48.

- Zantop, Thore, Wolf Petersen, and Freddie H. Fu. 2005. "Anatomy of the Anterior Cruciate Ligament." *Operative Techniques in Orthopaedics* 15(1): 20–28.
- Zhou, Shaobing et al. 2007. "Hydrogen Bonding Interaction of Poly(D,L-Lactide)/Hydroxyapatite Nanocomposites." *Chemistry of Materials* 19(2): 247–53.
- Zigdon-Giladi, Hadar et al. 2017. "VEGF Release from a Polymeric Nanofiber Scaffold for Improved Angiogenesis." *J Biomed Mater Res Parte A*: 2712–21.
- A.Srivastava, P. Jaiswal, "Bone Grafts and Substitutes Market by Type and Application - Global Opportunity Analysis and Industry Forecast, 2018-2025", Market Research Report, September 2018.
- "Global Bone Replacement Products Market, by Product Type, Material, End User, Region and Geography - Global Trends and Forecast to 2025", Market Research Report, November 2017.
- "Bone Grafts and Substitutes Market Size, Share & Trends Analysis Report by Material Type, By Application Type, By Region, And Segment Forecasts, 2018 – 2025", Market Research Report, June 2018.
- "Anterior Cruciate Ligament (ACL) Injuries-OrthoInfo - AAOS". orthoinfo.aaos.org. March 2014. Archived from the original on 5 July 2017. Retrieved 30 June 2017.