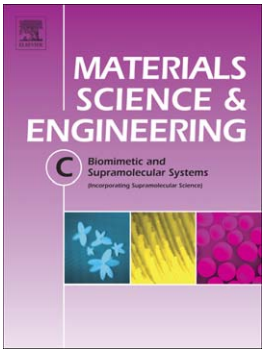

Bone Scaffolds with homogeneous and discrete gradient mechanical properties

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Bone Scaffolds with homogeneous and discrete gradient mechanical properties

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Abstract

Bone TE uses a scaffold either to induce bone formation from surrounding tissue or to act as a carrier or template for implanted bone cells or other agents. We prepared different bone tissue constructs based on collagen, gelatin and hydroxyapatite using genipin as cross-linking agent. The fabricated construct did not present a release neither of collagen neither of genipin over its toxic level in the surrounding aqueous environment. Each scaffold has been mechanically characterized with compression, swelling and creep tests, and their respective viscoelastic mechanical models were derived. Mechanical characterisation showed a practically elastic behaviour of all samples

and that compressive elastic modulus basically increases as content of HA increases, and it is strongly dependent on porosity and water content.

Moreover, by considering that gradients in cellular and extracellular architecture as well as in mechanical properties are readily apparent in native tissues, we developed discrete Functionally Graded Scaffolds (discrete FGSs) in order to mimic the graded structure of bone tissue.

These new structures were mechanically characterized showing a marked anisotropy as the native bone tissue. Results obtained have shown FGSs could represent valid bone substitutes.

Keywords:

bone scaffold, hydroxyapatite, genipin, mechanical properties, gradient

Introduction

Many surgical cases require reconstruction of bone defects, that may be congenital or induced by a disease or a trauma. The traditional methods of bone-defect reconstruction include autografting and allografting cancellous bone, applying vascularized grafts of different bone site, such as fibula and iliac crest. Bone grafting can induce functional bone remodeling, and morbidity of the harvesting site [1-5]. Moreover there is a limit on amount of usable material and possible mismatches between graft and defect shape. Furthermore, since bone grafts are avascular and dependent on diffusion, the size of defect and viability of host bed can limit their application. New bone volume maintenance can be problematic due to unpredictable bone resorption (in large defects, grafts can be resorbed by external environment before osteogenesis is completed [6-7]). Allografting introduces the risk of disease and/or infection, vascularized grafts requires sophisticated surgical methods and distraction osteogenesis techniques are reserved only for most motivated patients [8-9].

On the other side, synthetic material reconstruction has no limit on usable material, and shapes can be specifically manufactured for a given reconstruction. However, synthetic materials do not integrate perfectly with biological tissue, inducing an inflammatory response due to stress-shielding effect [10].

Although previous reconstruction methods have been successfully applied in many applications, their shortcomings have motivated a novel approach, called bone tissue engineering (bone TE).

Bone TE uses a scaffold either to induce bone formation from surrounding tissue or to act as a carrier or template for implanted bone cells or other agents. The scaffold provides an initial support structure that slowly degrades as healing bone tissue gradually regenerates. Scaffolds must be biocompatible, non-immunogenic, non-toxic and absorbable, with an absorption rate

similar to that of new bone formation. Materials used as bone tissue-engineered scaffolds may be injectable or rigid. Injectable materials (small particles or semi-liquid polymers that can be cross-linked in situ) are preferable for irregular defects reconstruction, while solid materials are more appropriate for large bone defects [11]. Materials commonly used are metals, ceramics, natural or synthetic polymers and composites [12-16]. Hydroxyapatite, calcium phosphate and a wide variety of ceramic matrices are appropriate for cell transport as they stimulate their differentiation and bone growth. However, there are problems associated with biodegradability, inflammatory and immunological reactions when they are used as carriers of osteoinductive factors. To overcome these drawbacks, synthetic biodegradable polymers based on polylactic acid (PLA), polyglycolic acid (PGA) and their polylactic-co-glycolic acid copolymers (PLGA) have been developed [17].

Polymers with an erodible surface (e.g. poly-ortho esters) may be beneficial in load bearing bone applications because only the surface undergoes degradation, leaving the material that provides mechanical strength, reducing the risk of implant failure [18]. In addition to appropriate mechanical properties, the scaffold must also have the right internal micro-architecture with interconnected pores of 200-400 μm diameter (the average size of the human osteon is approximately of 223 μm) [19]. Pore size is known to affect cellular affinity and viability by influencing cellular movement, binding and spreading, intracellular signaling, and transport of nutrients and metabolites [20].

Moreover, because concentration gradients of bioactive signaling molecules guide tissue formation and regeneration, and gradients in cellular and extracellular architecture as well as in mechanical properties are readily apparent in native tissues, it is important to consider this aspect in attempting to regenerate tissue by incorporating gradients into engineering design strategies [21-23].

This paper presents the preparation of polymeric support structures (scaffolds) that are able to guide growth and organization of seeded cells (either differentiated or stem cells) toward bone phenotype. The scaffolds are composed of a natural matrix made of gelatin embedding hydroaxypatite, to furnish the right mechanical properties to them, and collagen that acts as chemio-attractor for cells. Different concentrations of the three components are made. Because gelatin matrix without no cross-linking treatment solves in cell medium culture, genipin is used a natural cross-linker. Genipin was chosen among other cross-linking agents to show the presence of a proteic component up to the inner parts of the final samples, thanks to the blue colouring obtained by spontaneous reaction of genipin with amino acids and proteins. Moreover, genipine-fixed tissue has a resistance against enzymatic degradation comparable to the glutaraldehyde-fixed tissue but genipin is less citotoxic than glutaraldehyde [24,25].

Each construct has been mechanically characterized with compression, swelling and creep tests, and their respective viscoelastic mechanical models have been derived. Furthermore, since collagen must remain within the construct to act as chemo-attractive factor promoting cell colonization, its release in a surrounding aqueous environment has been assessed by UV spectrophotometry. Moreover an excessive quantity of genipin causes toxic effects [26], also its concentration in the surrounding aqueous environment has been evaluated. Scaffold morphology was evaluated by SEM analysis, while a qualitative-quantitative analysis of materials distribution was obtained with SEM+EDX analysis. Preliminary cellular tests were also performed, to evaluate cell vitality of cells seeded onto the scaffolds.

Then, taking into account that bone tissue has a gradient structure in terms of mechanical properties and porosity, we developed discrete Functionally Graded Scaffolds (discrete FGSs) that presented those features within their volume. They were prepared by stacking the composites described in the first part of this paper, then they were mechanically characterized and modeled.

Discrete FGSs morphology has been evaluated through SEM analysis. In this way, we have developed a simple method to prepare bioactive graded structures that can be used in bone tissue engineering applications.

MATERIAL AND METHODS

Genipin cross-linked gelatin

Genipin (GP) was added as cross-linking agent to a 10% w/v gelatin solution (Type A gelatin, Sigma Aldrich) in a 0.5% w/v ratio (respect to the gelatin solution volume). This solution was stirred at 50°C until the cross-linking process started (after 30 minutes). Then, the solution was cast in a Petri dish and left at room temperature for 48 hours in order to complete the cross-linking process. Other samples were obtained leaving the solution at room temperature for 10 days. Both kinds of samples were mechanically tested.

Homogeneous composites

Different homogeneous samples were prepared, varying hydroxyapatite (HA) concentration from 30 to 90% w/v, respect to whole volume of liquid components. For each concentration, samples were prepared according to two different protocols that differ only in the sonication process, finalized to better fragment and disperse HA crystals within hydrogel matrix. A Vibra-CellTM (Sonics & Materials, CT, USA) ultrasonic probe was used for this purpose. In both cases, the components of the samples are the following:

- 10% w/v gelatin solution in milliQ water;
- PBS (Phosphate Buffered Saline) solution, added in a 1:1 volume ratio, respect to the gelatin solution volume;
- 1.2% w/v collagen solution, added in a 2:1 volume ratio, respect to the gelatin solution volume;
- HA, properly added in order to obtain composites with HA concentrations ranging from 30 to 90% w/v, respect to whole volume of liquid components;

- GP, added in a 0.5% w/v ratio, respect to the whole volume of liquid components.

The first series of samples (protocol 1) were prepared mixing HA powder within the PBS solution at room temperature, then sonicating this mixture at 0.2V for 2 minutes. 1.2% w/v collagen solution was added to the gelatin solution in a 2:1 volume ratio, and finally the previous prepared HA/PBS mixture was added in a 1:1 volume ratio respect to gelatin solution volume. At the end, GP was added to all mixture in a 0.5% w/v ratio, respect to the whole volume of its liquid components. The final mixture was stirred at 50 °C until the beginning of cross-linking process (about 40 minutes, depending on HA concentration. Higher HA concentrations stretch this time, because a greater quantity of HA interferes with the cross-linking process).

The other protocol (protocol 2) provides the sonication of whole mixture, rather than just HA/PBS ones. So all components were mixed according to previous ratios and the final mixture was sonicated for 2 minutes at 0.2 V.

In both protocols, samples were left at room temperature either for 48 hours or 10 days.

Discrete graded scaffold were prepared stacking mixtures with different HA concentrations, obtained as previously described. Precisely, three different discrete gradient samples were prepared, stacking different couple of homogeneous layers characterized by following HA concentrations (percentages are to be intended as weight of HA respect to all volume of liquid components): 30-50%, 60-70%, 80-90%. The genipin cross-linking process was used to join the two different layers. First, the bottom layer with desired HA concentration was prepared, than the second layer (with a lower HA concentration) was cast on it only when genipin cross-linking process started, thus avoiding mixing between layers and allowing, at same time, a parallel cross-linking completion that joins the two different layers.

Each two-layer scaffold was prepared according to the protocol 2, as homogenous samples obtained with this protocol presented better mechanical properties than the other one (see results section).

Collagen release assessment

Initially a calibration curve was obtained measuring the absorbance of different concentration of collagen solutions (between 0.0001 until 2% w/v) through UV spectrophotometry (BMG Labtec, Italy) at 225nm. The amount of collagen released from samples into the surrounding aqueous environment was investigated. The samples (6 x 6 mm square with a thickness of 2 mm) were completely immersed in a Petri dish containing 20 ml of PBS solution. At different times (1m, 2m, 5m, 10m, 15m, 30m, 1h, 2h, 4h, 8h, 24h) 0.2 ml of water bath was sampled and analyzed in absorbance at 225 nm with an UV-vis spectrophotometer (OMEGAstar - BMG Labtech, Italy) to assess the amount of released collagen.

GP release assessment

Also in this case a calibration curve was obtained measuring the absorbance of different concentration of genipin solutions (between 1 μg/ml until 5 g/ml) through UV spectrophotometry (BMG Labtec, Italy) at 238 nm. According to Liu et al. prepared scaffolds (6 x 6 mm square with a thickness of 2 mm) were dipped in 20mL of PBS solution and incubated at 37°C to evaluate release of unreacted genipin (GP) [27]. 200 μl of soaking solution were daily collected for 7 days and analyzed using an ultraviolet-visible light spectrophotometer (OMEGAstar - BMG Labtech, Italy) at 238nm their absorbance was obtained.

Load-unload cyclic tests

Load-unload tests were conducted using Zwick/Roell mod. Z005 (Zwick GmbH & Co, Germany) controlled in position. The samples (6 x 6 mm with a thickness of 5 mm) were subjected to two consecutive compressive load-unload cycles at T=25 °C with the following settings:

- strain rate: 0.07 mm/s;
- end of loading phase: 15% strain (respect of the initial sample length);
- end of load-unload cycle: no load.

Then elastic moduli were calculated from the first linear tract of stress-strain curve for each cycle. Data were processed and results are reported as mean ± standard deviation (n=3).

Swelling and creep tests

These tests were performed with the Ugo Basile 7006 isotonic transducer (Ugo Basile, Italy) connected to the MP35 acquisition platform (BIOPAC Systems Inc, Italy). The swelling test allows the evaluation of the swelling of a sample dipped in water. Once the initial length l_0^{real} of the sample was measured, the latter was placed in a Petri dish and mounted on the transducer, so its deformations were measured. The sampling rate was fixed at 1 Hz. After one minute of acquisition with no load, the offset was evaluated and the Petri plate was filled with deionized water until the sample was completely covered. So, the measured time-dependent deformation was equal to:

$$\varepsilon(t) = \frac{l^{real}(t) - l_0^{real}}{l_0^{real}} \qquad \text{(eq 1)}$$

where $l^{real}(t)$ is obtained by adding the offset to the measured length $l(t)$, so it represents the elongation of the sample at time t.

The test was performed until a stable plateau was reached, so the swelling time constants were derived for each sample through the analysis of the collected data.

The swelling was also measured weighing the sample before to deep in water and then at different times (1m, 5m, 10m, 15m, 30m, 1h, 2h, 4h, 8h, 24h). The sample was removed from the bath, dried and weighed again.

Creep test was performed in aqueous bath after the swelling test on each sample, so as to have stabilized its original length. In this way the initial length of the sample was equal to:

$$l_{0,creep}^{real} = l_0^{real}\left(1+\varepsilon_{endswelling}\right) \qquad \text{(eq.2)}$$

where l_0^{real} ,is the initial length of sample and $\varepsilon_{endswelling}$ was the deformation reached in the plateau area after swelling. The sampling frequency was fixed at 2 Hz. The load necessary to cause 1% linear deformation of a sample was derived from its stress-strain graph.

SEM, SEM-EDX and porosity measurement

SEM (Scanning Electron Microscopy) and SEM-EDX (Scanning Electron Microscopy - Energy Dispersive X-ray) micro-analysis were performed only on dried samples, that were mounted on aluminium stubs and then covered with Au by the Edwards Sputter Coater B150S equipment. Samples were observed with a Philips XL20 microscope (Royal Philips Electronic, Eindhoven, The Netherlands). SEM-EDX is an analytical technique used for the elemental analysis or chemical characterization of a sample.

Using a purposely software developed in Matlab® (The Mathworks Inc., MA, USA) environment, pictures from SEM analysis were processed and porosity, as ratio between void space and composite volume, was measured.

Cell culture

MG-63 human osteoblast-like cells (ATCC, Rockville, MD) were grown in a controlled atmosphere (5% CO_2; T=37°C) in DMEM supplemented with 10% foetal bovine serum (FBS, Gibco, Italy), 1% L-

glutamine, and 1% antibiotics (penicillin-streptomycin - Gibco, Italy). After thawing, they were routinely split 1:10 every 2-3 days and used at the 4th passage.

Before seeding the samples were sterilized in ETOH 70% for 30 min, washed in PBS and incubated with DMEM and 10% FBS for 3 hours. The medium was then discarded and MG63 cells were detached using 0.25% trypsin in 1mM EDTA and plated (in triplicate) onto the samples and in 24-well polystyrene tissue culture plates (TCPs) as controls at a density of $1x10^4$ cells/cm^2. As control wells only coated with 0.1% gelatin solution in deionised water and then washed before cell seeding were used.

Cell Viability Assay

After incubation (24, 48 and 72 hours), the culture medium was removed from each well; 200 µl of CellTiter- Blue Reagent (0.1 mg/ml in DMEM without phenol red) was added to each sample; the multi-well plates were incubated at 37°C for 4h. Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of viability and cytotoxicity. After that time, the multiwell was read spectrophotometrically (Tecan Infinite M1000,Tecan, USA) at 560 nm and 690 nm and cell viability was quantified.

Because genipin may interfere with results, the fluorescence of a sample without cells was valuated for each concentration. This value was then subtracted to the fluorescence value of samples seeded with cells.

The measured values are expressed as a percentage over control cultures (TCPs) and the data reported here represent means and standards deviations obtained from at least three different experiments.

RESULT AND DISCUSSION

Collagen release assessment

Spectrophotometric analysis shows that no collagen is released from the scaffold matrix into the surrounding aqueous environment. According to this evidence, no graphs are showed in this paper. From this result it is possible to hypothesized that part of collagen react with genipin crosslinking its fibers and also create covalent bonding with gelatin matrix, for this reason remains anchored inside the polymer matrix.

Genipin release assessment

Results shows that GP is entirely released in the first day, then its concentration in bath solution remains constant (Fig. 1a-b). Since maximum concentration of GP is equal to 35 μg/ml in bath solution (HA 90% w/v, protocol 1), it is possible to infer that they are biocompatible, because GP becomes cytotoxic only when its concentration exceeds 80 μg/ml. In addiction, our experimental results confirm those obtained by Sung et al [27]. According to them, GP concentration in aqueous solution tends to decrease with time and this behavior was more pronounced with increasing temperature and pH.

Load-unload cyclic tests

GP cross-linked gelatin

Table 1 shows elastic moduli of GP cross-linked gelatin samples at 48h and 10 days after their preparation. In both cases there was an increase of the curve slope in the second load-unload cycle due to phenomena such as expulsion of residual water (especially for samples tested 48 h after their preparation) and collapse of inner pores. The increased time of casting increased stiffness of GP cross-linked gelatin: elastic moduli after 10 days result double respect of samples

tested 48 h after preparation. In fact, after 10 days, all the water present in the samples is evaporated and gelatin is organized in a stable and rigid structure.

Composite homogeneous samples

As in the case of GP cross-linked gelatin, composite samples, prepared according both protocol 1 and 2, have been mechanically tested 48h and 10 days after their preparation. Furthermore, anisotropy has been assessed in samples with 30% and 50% w/v HA, applying a load on both their top and lateral surfaces. Results are summarized in histograms.

48h after preparation

Elastic moduli of samples prepared according to protocol 1 and 2, are shown in Fig. 2a-b. Compressive elastic modulus basically increases as content of HA increases and it is always greater in the second load-unload cycle especially due to collapse of internal pores and expulsion of residual. In general, elastic modulus of samples prepared according to protocol 2 results bigger than one of protocol 1 for each HA concentration; in addiction, data from protocol 2 samples are more homogeneous (the standard deviation for each group is smaller).

Mechanical anisotropy has been assessed in samples with 30% and 50% w/v HA prepared according protocol 2. Results are shown in Fig. 3a-b, distinguishing between normal and tangential direction of compression. Similarly to previous results, the elastic modulus increases with the content of HA and it is always greater in the second cycle. Only the samples with 50% (w/v) HA show a marked anisotropy, resulting more rigid in the tangential direction.

10 days after preparation

Elastic moduli for samples prepared according protocol 1 and 2, are shown in Fig. 4 (a-b). In this case it is necessary to do some difference between the two protocols. For protocol 1, compressive elastic modulus basically increases as content of HA increases and it is always greater in the

second load-unload cycle especially due to collapse of internal pores. This is true until 90% of HA in sample, where there is no statistical difference between the two cycles. This is due because, being high the content of HA and the water completely evaporated, the inner pores during compression test rapidly collapse. Because the elastic component is not sufficient to recover the initial structure the two cycles do not give a statistical relevant difference in the elastic modulus. Instead, the samples prepared with protocol 2 present a light increase of elastic modulus as HA concentration increases and there is no difference between the two cycles. This results suggests that the water inside the samples plays a fundamental role in order to tune mechanical properties as shown in previous paragraph.

Studying mechanical anisotropy in 30% and 50% w/v HA samples (prepared according protocol 2) ten days after their preparation yielded the following results (Fig. 5 a-b).

The increase of the elastic modulus with HA content is confirmed, while there is no a statistical difference between the two cycle confirming the previous results. Again, only the sample with 50% (w/v) HA showed a marked anisotropy, resulting more rigid in the tangential direction.

Cyclic compression tests on discrete gradient samples

Previous results show that resulting stiffness of composite scaffold is mainly dependent on hydroxyapatite concentration. Therefore, a discrete gradient of stiffness has been obtained from a discrete gradient of HA concentration. In particular discrete gradient structure have been prepared stacking two different homogeneous layers (see Material and Methods section). Samples have been mechanically tested both 48h and 10 days after their preparation. Discrete gradient samples are intrinsically anisotropic, as their two layers react in series or in parallel to the load, depending on its direction of application. According to Figure. 6a, layers react in series when the load is applied in the normal direction (versor *n* in figure), while they react in parallel when construct is subjected to a tangential load (versor *t* in figure).

Results for discrete gradient scaffolds are shown as for previous composite homogeneous samples.

48h after preparation

Elastic moduli for layered structures are shown in the following figure 6 b-c-d. Results confirmed that compressive modulus increases as HA total concentration increases and it is always greater in the second cycle for both loading directions. Furthermore, discrete gradient structures were more rigid when their layers are subjected to a tangential load, as expected from theoretical model. This evidence was more pronounced within the second cycle, since both layers were stiff due to residual water expulsion and collapse of internal pores.

10 days after preparation

Elastic moduli for layered structures are shown in figures 7 a-b-c. Observations made to discuss previous results are again applicable. Note that the longer duration of the casting process involves a general tightening of the compression tested structures, as expected.

Swelling test

We did not observed appreciable swelling in aqueous environment for any sample. According to this evidence, no graphs are showed in this paper. The measurement obtained using isotonic transducer confirmed the results obtained with classic method.

Creep test

Tested materials showed a mechanical behaviour similar to the Kelvin model, that it is expressible with the following relationship:

$$\varepsilon = \varepsilon_{spring} + \varepsilon_{Voigt} = \frac{\sigma_0}{k_1} + \frac{\sigma_0}{k_2}\left(1 + e^{-t/\tau}\right) \qquad \text{(eq 3)}$$

This model fits very well the experimental data collected. It is possible to note that samples have principally an elastic behaviour and low viscosity constants and, consequently, their characteristic creep time is short: the average considering all samples is 4.3 ± 0.5 s.

Porosity estimation and fitting of the experimental elastic moduli data

Theoretical composite elastic moduli in compression can be calculated using models reported in literature. According to Gibson and Ashby [28] the overall elastic modulus of a composite including a volumetric fraction f_i of particulate can be evaluated as:

$$E_0 = \frac{5(E_i - E_m) f_i}{3 + 2\frac{E_i}{E_m}} + E_m \qquad \text{(eq.4)}$$

where E_i and E_m are the elastic moduli of inclusion and matrix, respectively. Note that this model does not contemplate any porosity of construct, so theoretical calculated values must be corrected by the actual porosity of prepared structures. E_m (i.e. GP cross-linked gelatin elastic modulus) is experimentally derived, while E_i (i.e. hydroxyapatite elastic modulus) has been taken from literature. f_i is calculated on the basis of dry volume of the main components of the composite constructs (i.e. gelatin and HA), considering ρ_{HA} = 3.16 g/cm^3 e $\rho_{gelatin}$ = 1.34 g/cm^3 as inclusion and matrix densities, respectively. For example, 4ml mixture of the 60% w/v HA composite contains 2.4g HA and 0.10g gelatin (see Materials and Methods section), so the volumetric fraction of the inclusion can be calculated as:

$$f_i = \frac{v_i}{v_m + v_i} = \frac{m_{HA}/\rho_{HA}}{m_{gelatin}/\rho_{gelatin} + m_{HA}/\rho_{HA}} \cong 0.911 \qquad \text{(eq.5)}$$

Table 2 shows inclusion volumetric fractions and theoretical overall compressive moduli (calculated according eq. 5) for samples with an HA content varying from 60% to 90% w/v.

As anticipated, E_0 must be corrected by the actual porosity of the prepared structures. In this regard, the most commonly used formulas are the following:

$$E = E_0\left(1 - a \cdot p + b \cdot p^2\right) \quad \text{(eq.6)}$$

$$E = E_0\left(1 - p\right)^{\gamma} \quad \text{(eq.7)}$$

where *p* is the porosity of composite structure and E_0 is its elastic modulus when $p = 0$, calculated according [35]. The former formula has been used by McKenzie in 1950 [29] to model the compact bone stiffness and requires the estimation of two parameters (*a* and *b*), while equation 7 needs the estimation of only one parameter (γ) by fitting experimental data.

By definition, porosity or total pore volume fraction (*p*), whether open or closed, is given by the ratio between volume occupied by pores and geometric volume of the sample:

$$p = \frac{V_g - V_s}{V_g} \quad \text{(eq.8)}$$

The pore volume is estimated subtracting the volume of solid components from the geometric one. The first can be evaluated considering density and concentration of solid components in a manner similar to that previously used to estimate the volume fraction of the inclusion, while the second one can be obtained directly by measuring dimensions of the sample. Table 3 shows the estimated (eq. 8) and measured porosity (from SEM photos) for samples with an HA content varying from 60% to 90% w/v: it is possible to note there is a good accordance between them, so this theoretical method allows to predict the porosity of a scaffold with a reduction of experimental time and costs.

MATLAB® Statistic Toolbox (The Mathworks Inc., MA, USA) has been used to fit experimental values of elastic moduli with chosen theoretical models. In particular, since the error associated with experimental data is not constant (e.g. data were obtained averaging a different number of experimental measurements) we used a weighed fitting. In this regard, experimental values of

elastic moduli are weighted by their standard deviations. Only the exponential model (eq. 7) is suitable to fit the experimental data (table 4), while McKenzie's one (eq. 6) provides no acceptable result for its parameters.

SEM and SEM-EDX analysis

All samples are uniform in the section, demonstrating that the protocol 2 allows a perfect homogenization of components and there is not a phase separation inside them. Moreover, SEM analysis shows that increasing HA content, its crystals coats gelatin matrix and collagen fibers (Fig. 8a-b-c). In particular collagen fibers interconnect the HA crystals and acts as a glue between them. Moreover, the samples present a porous structure with a porosity decreasing as HA increasing. EDX analysis of homogeneous constructs indicates a uniform distribution of calcium (red, in Fig. 8b) and phosphorus (green, in Fig. 8b). The quantities of the elements, given as weight percentages) are Calcium = 70.28%, Phosphorous = 26.78 % and Magnesium = 2.94%. All these results give us a reasonable hope that the gelatin/HA construct with collagen as chemio-attractor could be a possible candidate for bone tissue regeneration.

Cell Viability

By analysing the vitality data obtained from cells seeded onto scaffolds (fig. 9), we observed that as HA concentration in composite structure increased cell viability decreased. Moreover, for each type of sample cell viability increased as cell culture time increased. These results that the structures were biocompatible and not toxic and also that the increase of HA induced a slower viability perhaps due to a minor porosity present in them. This test represented only a preliminary step toward more specific cell experiments in order to evaluate how these structure influenced cell activities.

Conclusions

In this paper we presented a new bone TE construct based on gelatin and HA. Genipin and collagen was used as cross-linker as chemio-attractor repsectively. We optimised also two possible protocols for their realisation. Samples, once formed, did not present neither collagen, neither genipin release over its toxic value. Mechanical tests of homogeneous composite bone substitute and of their stacks, designed to mimic the mechanical gradient structure of bone tissue, showed that they present a marked anisotropy as in native tissue.

On the basis of mechanical characterisation, some general conclusions can be outlined. The second of the two proposed protocols, which provides the sonication of all the components of the scaffold during preparation, gives better results in terms of homogeneity of the samples (smaller standard deviation). In addiction, it can be stated that compressive elastic modulus basically increases as content of HA increases, and it is strongly dependent on porosity as demonstrated by cyclic loading and confirmed by mathematical models. Another important factor is the water content: by decreasing the amount of water due to evaporation, elastic modulus increases.

Creep tests demonstrated that, regardless the HA concentration, samples showed an elastic behaviour. Based on SEM analysis, it was concluded that fabricated structures present an uniform distribution of calcium and phosphorous, and also that HA are interconnected each other by gelatin and collagen. These results suggest that these scaffolds present suitable features for their use as bone substitutes, in fact as reported by Even-Ram et al [30], the stiffness of substrate direct differentiation of stem cells toward specific fates, and in particular to address it toward bone fate the elastic modulus should be more than 100 kPa as in our case.

Preliminary cells tests on cell vitality were performed: results have shown that the structures were biocompatible and not toxic and also that the increase of HA induced a slower viability perhaps

due to a reduced porosity. For this reason future developments of this research will be in-vitro and in-vivo tests.

Figure legends

Figure 1: Genipin release in PBS during time, for various concentrations of hydroxyapatite and different preparation protocols, protocol 1 (a) and protocol 2(b).

Figure 2: Elastic moduli of samples prepared according to protocol 1 (a) and 2(b) for HA concentration from 60% to 90%, after 48 hours from preparation.

Figure 3: Elastic moduli of samples prepared according to protocol 2 for a concentration of HA of 30% and 50%, after 48 hours from preparation, along normal direction (a), and tangential direction (b) of compression.

Figure 4: Elastic moduli of samples prepared according to protocol 1 (a) and 2(b) for HA concentration from 60% to 90%, after 10 days from preparation.

Figure 5: Elastic moduli of samples prepared according to protocol 2 for a concentration of HA of 30% and 50%, after 10 days from preparation, along normal direction (a), and tangential direction (b) of compression.

Figure 6: (a) load application to FGSs: according to load direction (normal or tangential) the structures that form the scaffolds react as in series or in parallel; elastic moduli (after 48 hours from preparation) of FGSs made by homogeneous samples of 30/50% HA (b), 60/70 % HA (c) and 80/90% HA (d).

Figure 7: Elastic moduli (after 10 days from preparation) of FGSs made by homogeneous samples of 30/50% HA (a), 60/70 % HA (b) and 80/90% HA (c).

Figure 8: SEM micrographs at 250X magnification of 40% (a), 60 % and its SEM+EDX analysis (b) and 80% HA (c) samples. SEM+ EDX analysis calcium is indicated in red, whereas phosphorous in green.

Figure 9: Cell viability at 24, 48 and 72 hours onto four different types of scaffolds containing 30%, 50%, 70% and 90% w/v of hydroxyapatite respectively.

TABLES

Table 1

Elastic modulus	48 hours	10 days
E_{first_cycle}	0.32 ± 0.03 MPa	0.63 ± 0.11 MPa
E_{second_cycle}	0.39 ± 0.05 MPa	0.66 ± 0.09 MPa

Table 2

	60%	70%	80%	90%
f_i	0.911732	0.923375	0.932305	0.939371
E_0	2.15E+06	2.17E+06	2.18E+06	2.19E+06

Table 3

	60%	70%	80%	90%
Estimated	79%	76%	73%	70%
Measured	78%	77%	72%	70%

Table 4

	60%	70%	80%	90%
Γ	6.39	5.44	5.22	5.07

Tables legends

Table 1: Elastic moduli of GP cross-linked gelatin samples at 48 hours and 10 days after their preparation.

Table 2: Evaluated volume fraction and estimated elastic modulus for gelatin/HA homogeneous compounds (porosity in not taken into account)

Table 3: Estimated (eq. 8) and measured porosity for samples with an HA content varying from 60% to 90% w/v

Table 4: Exponential model (eq. 7) parameter values estimated using MATLAB® Statistic Toolbox

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Figure 1

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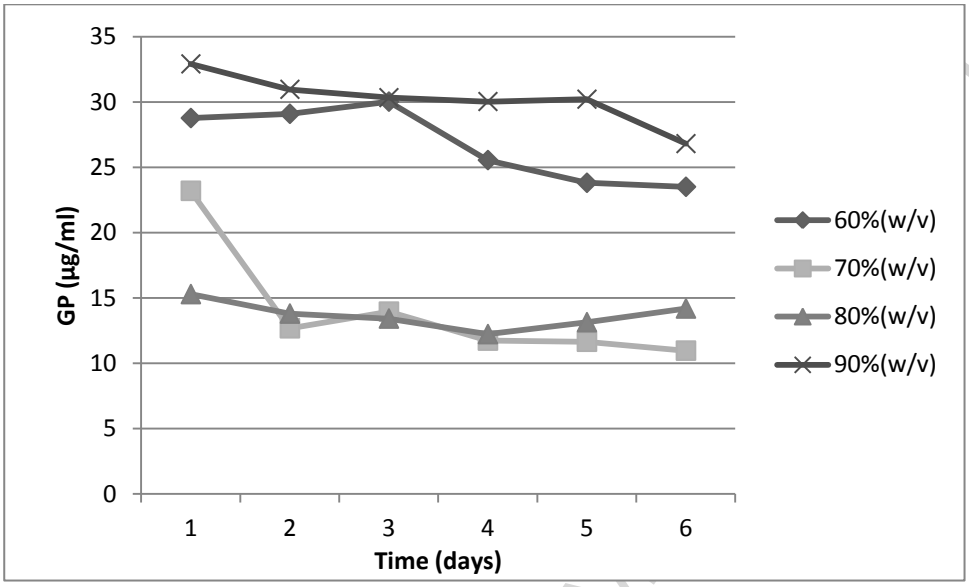

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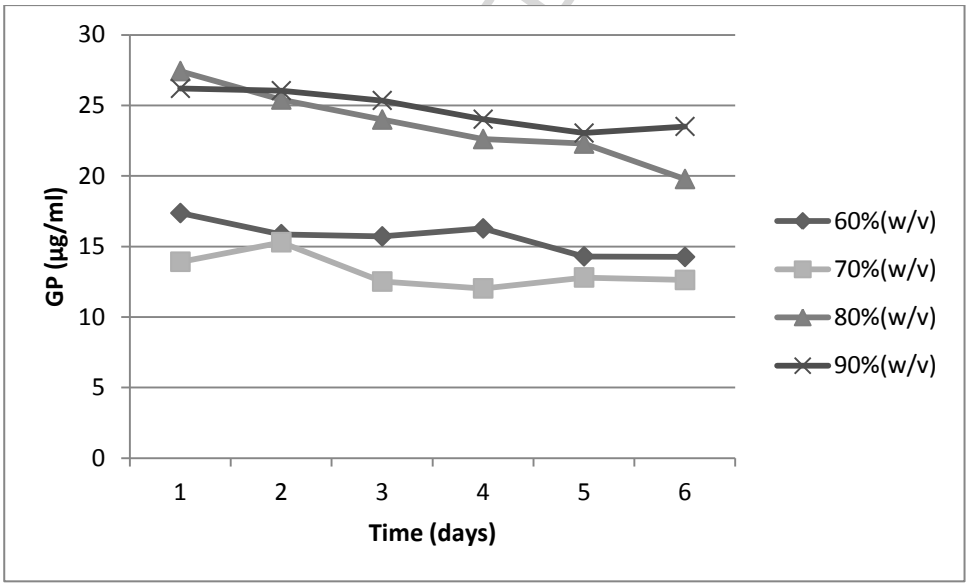

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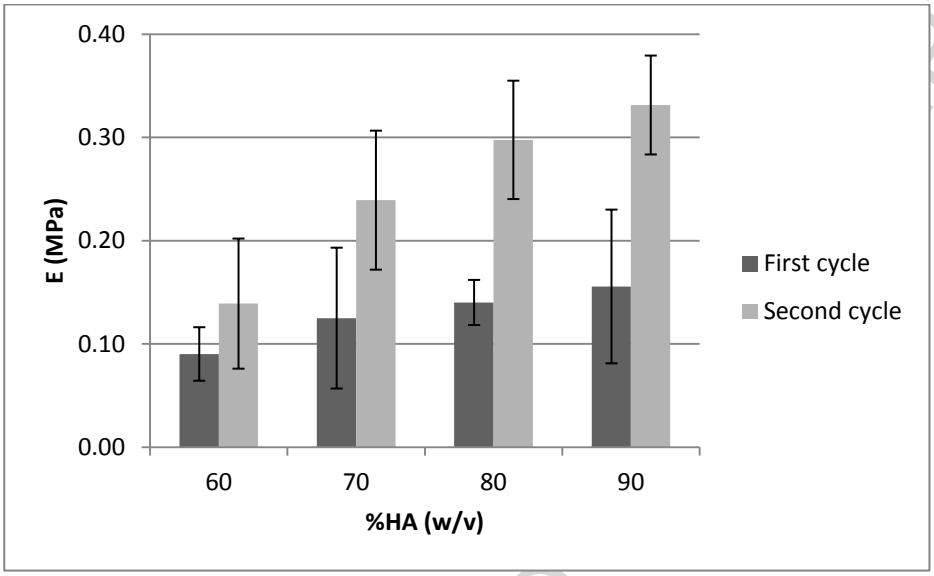

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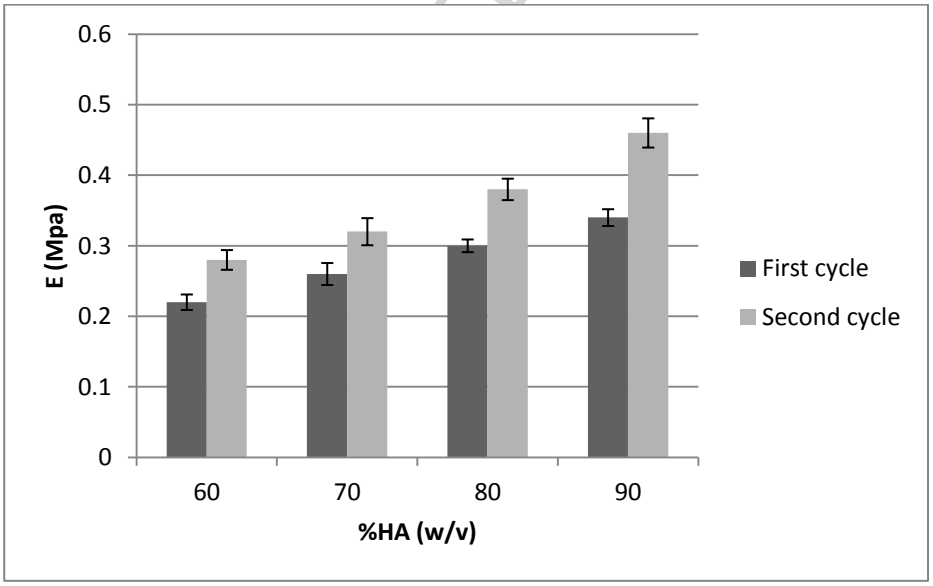

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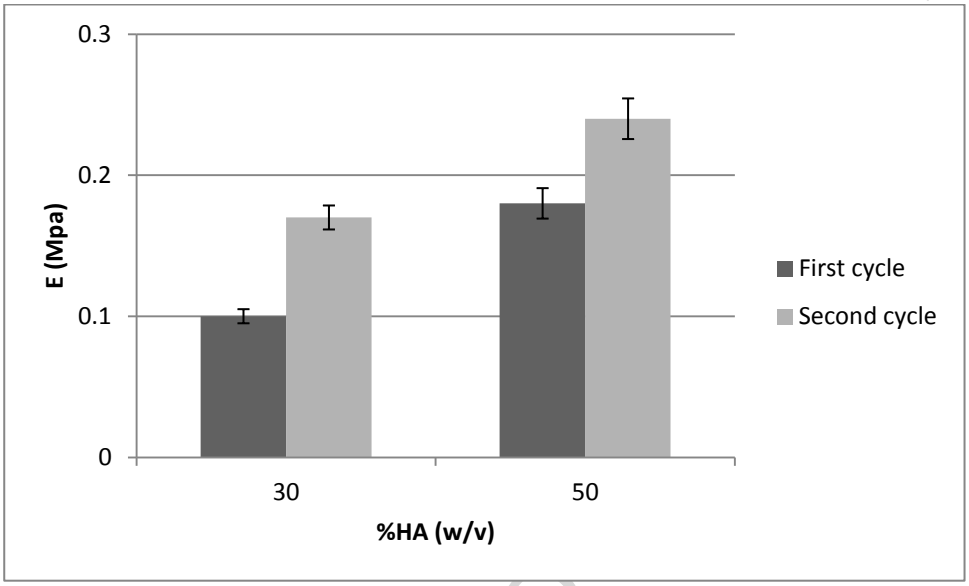

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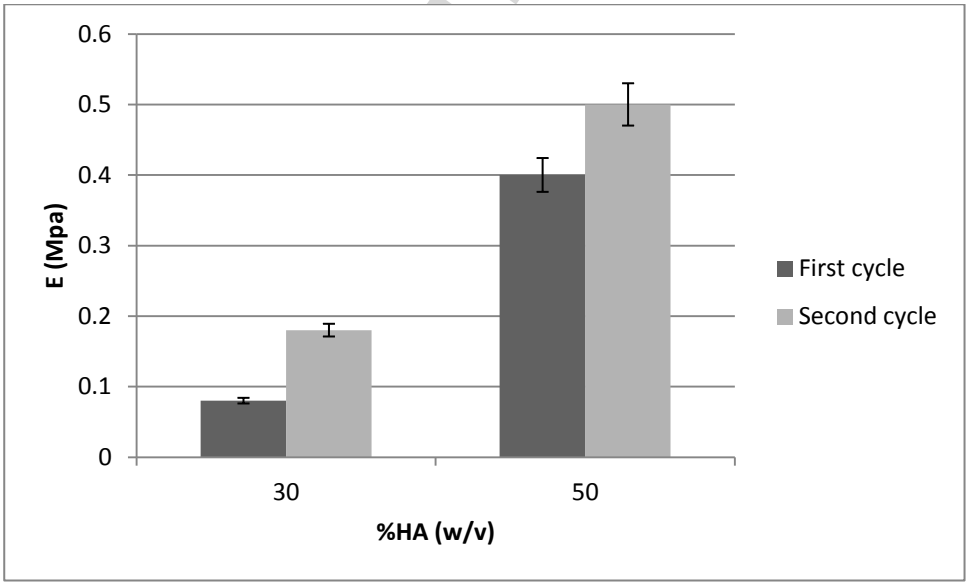

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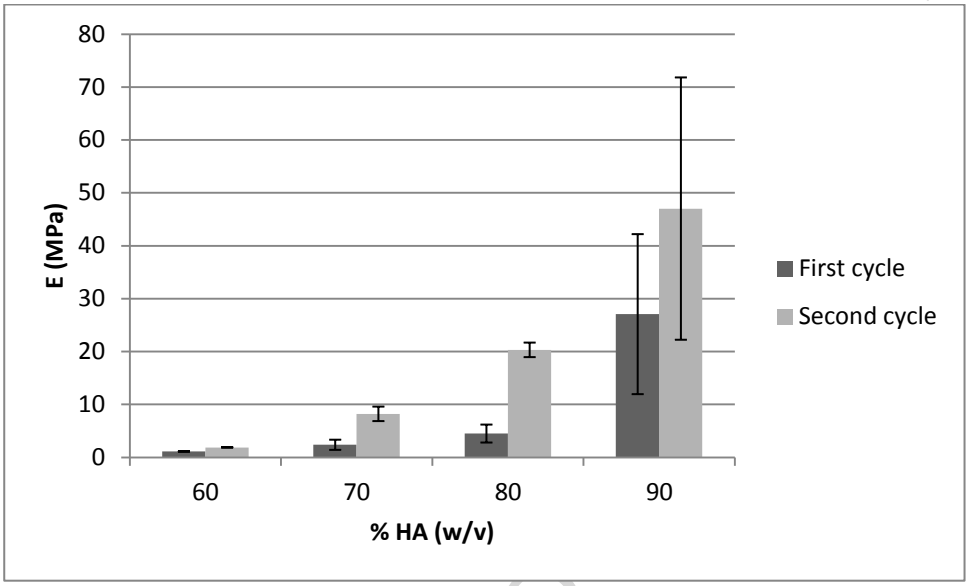

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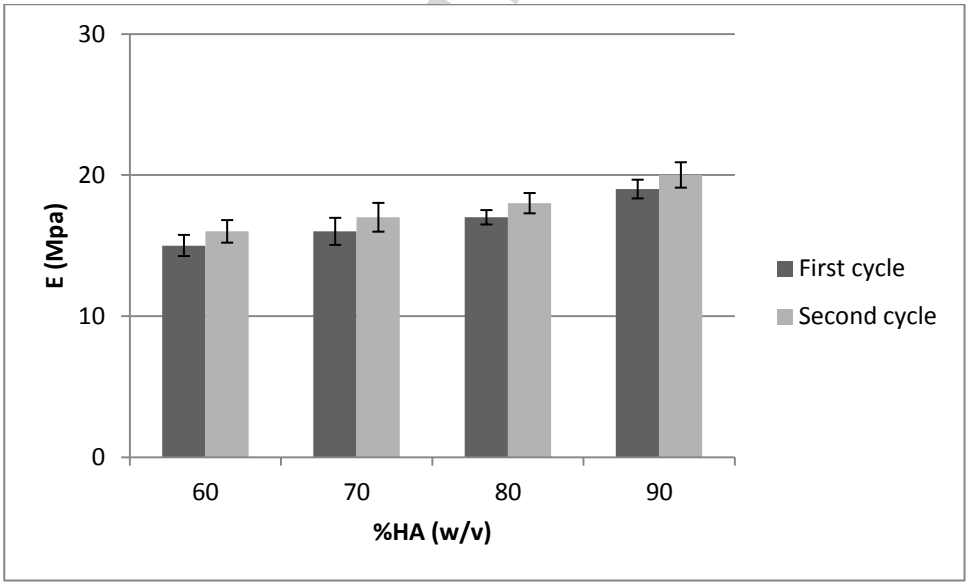

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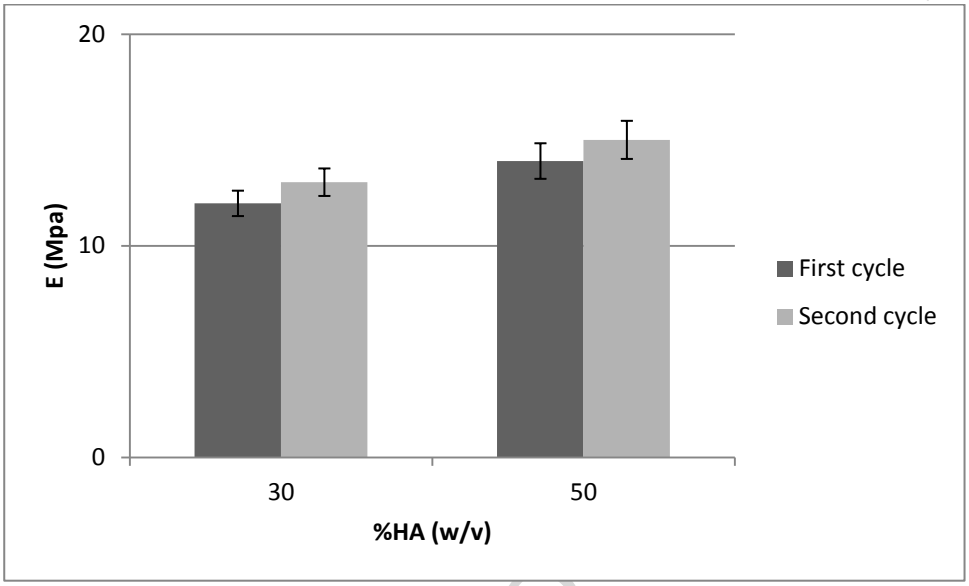

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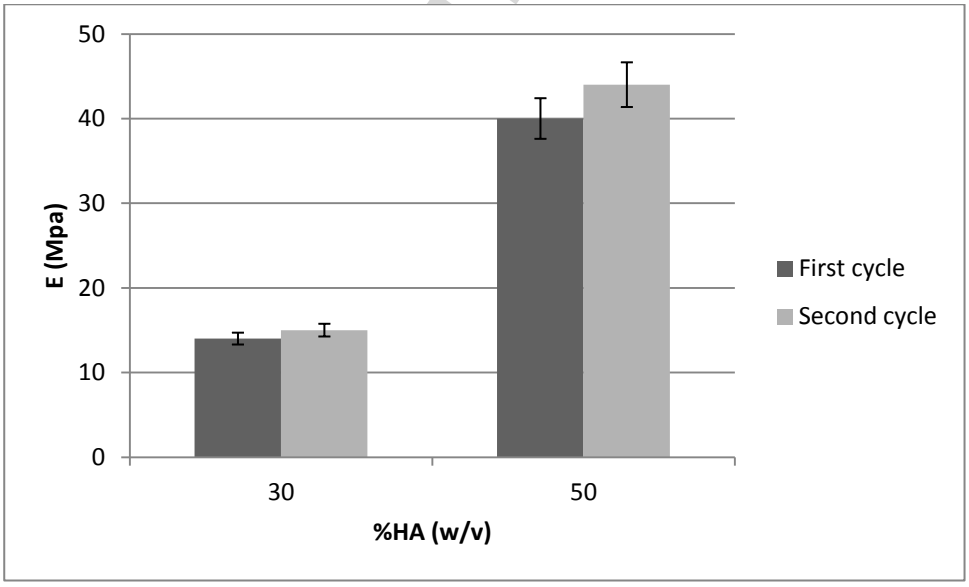

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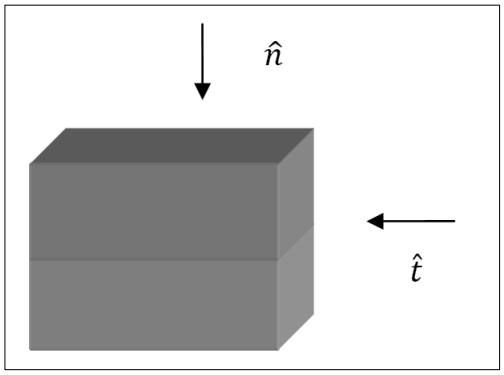

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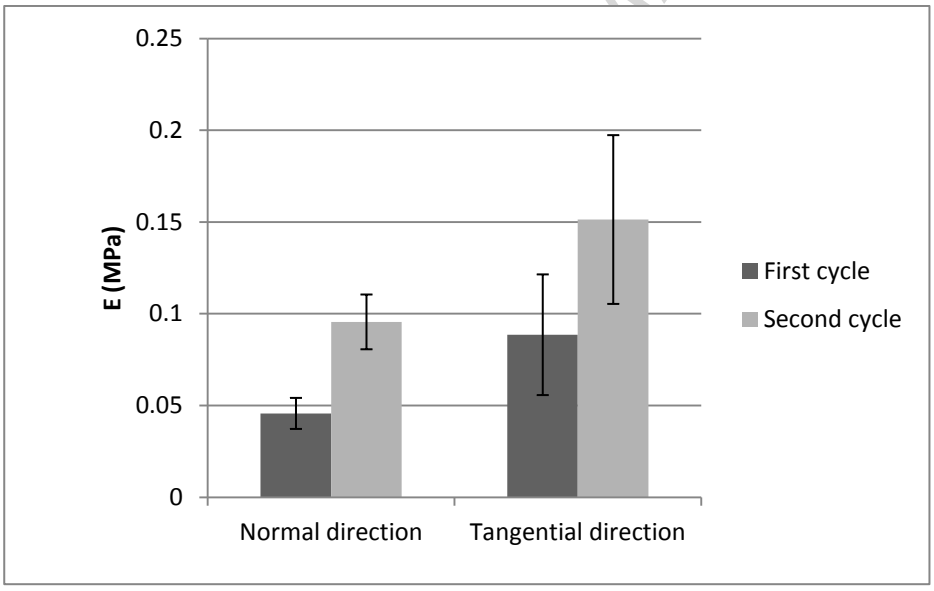

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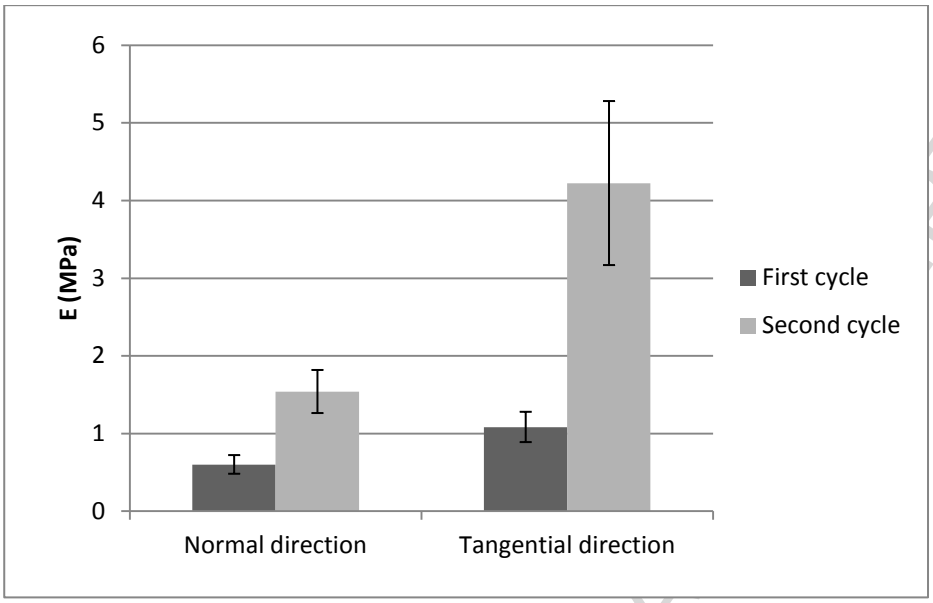

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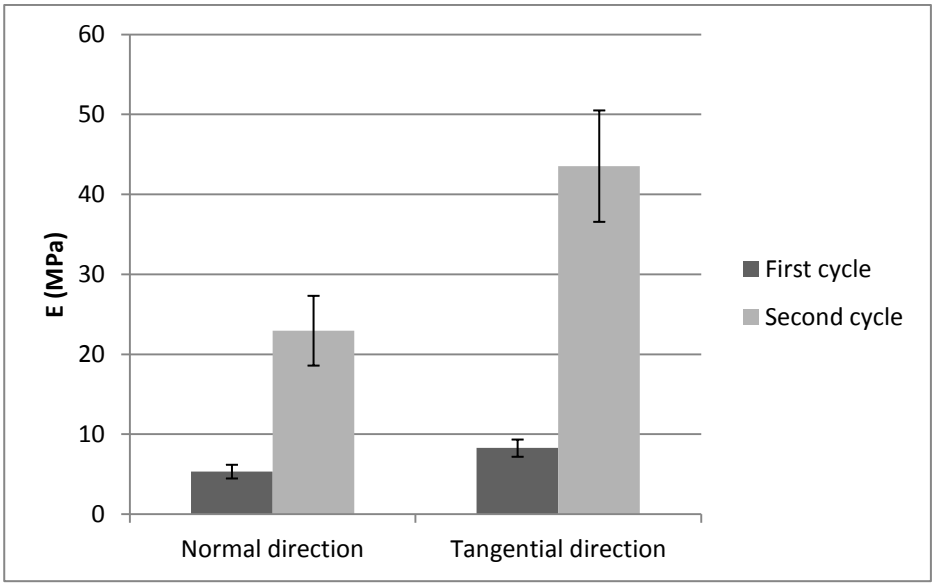

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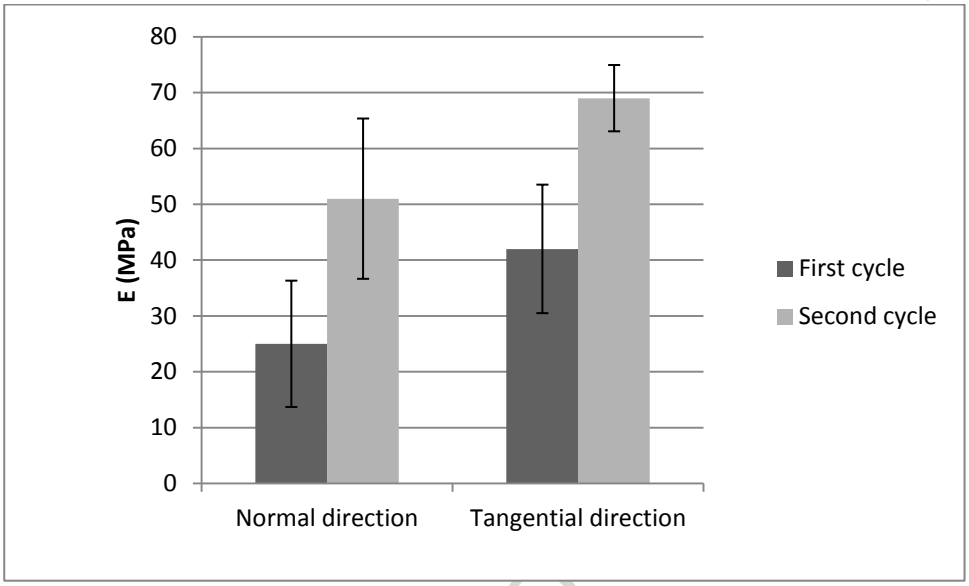

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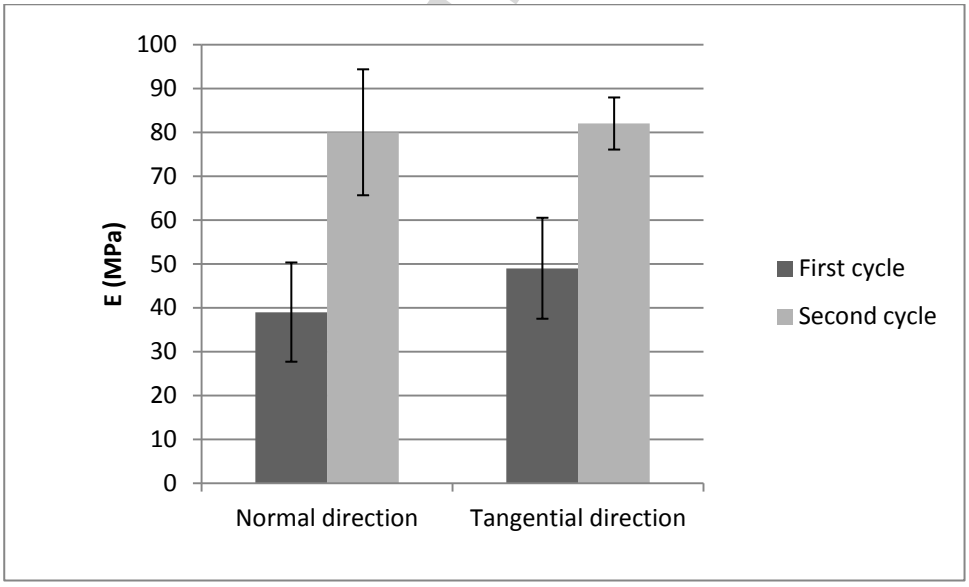

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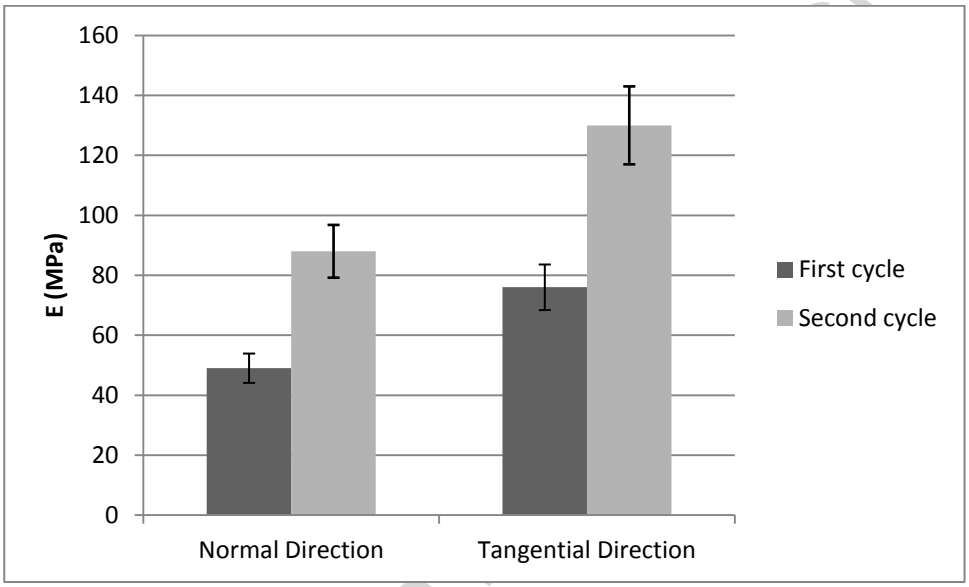

Figure 8

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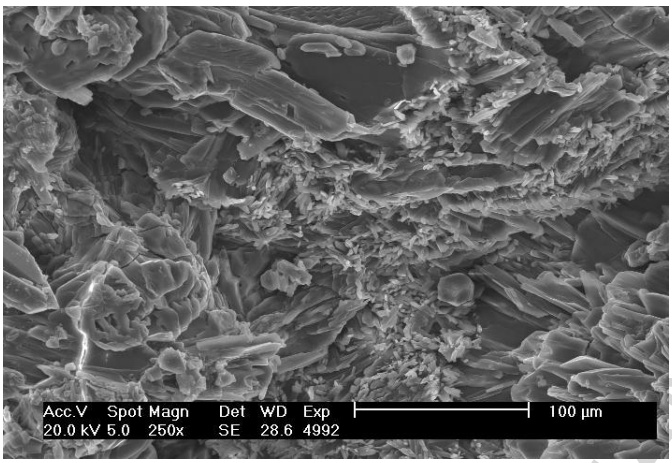

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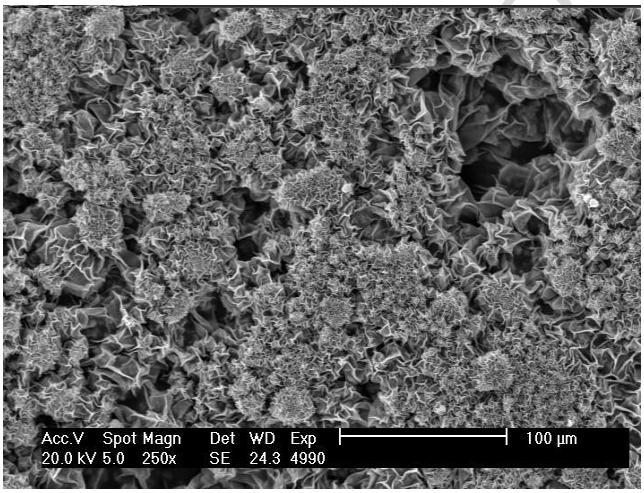

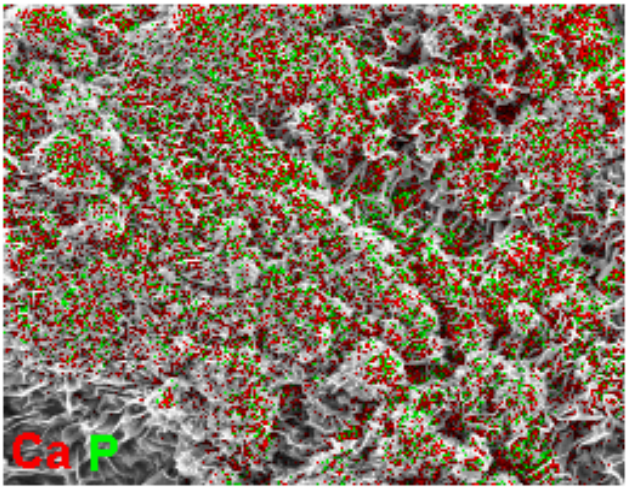

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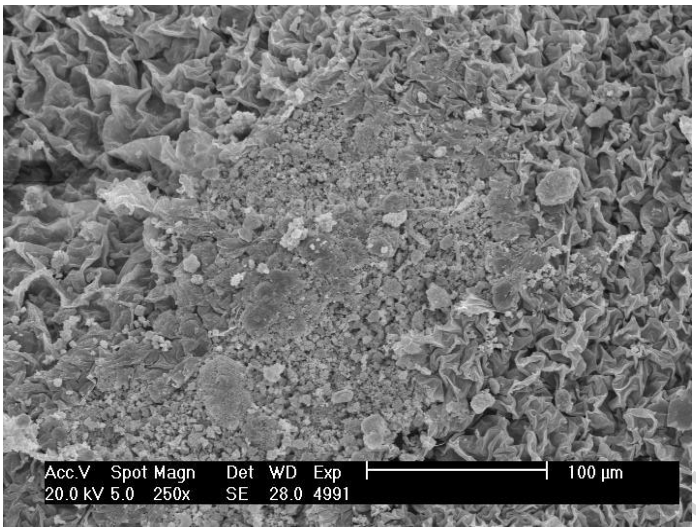

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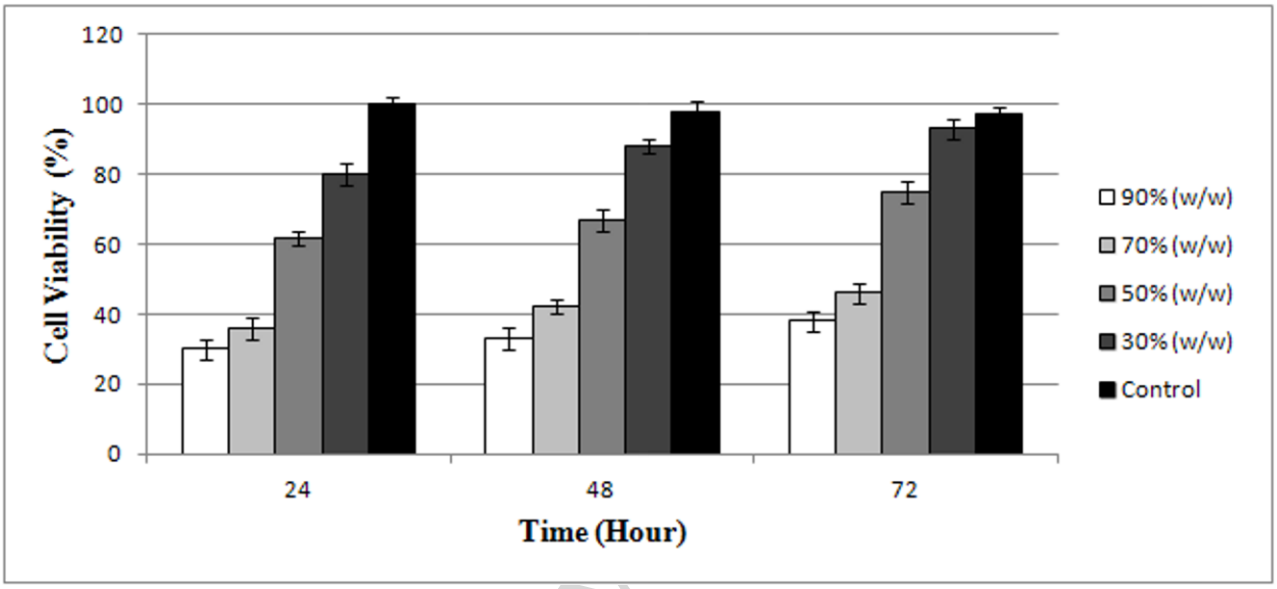

Highlights

- Bone tissue scaffolds based on collagen, gelatin, genipin and hydroxyapatite.
- Characterisation by mechanical test, genipin and collagen release test, SEM+EDX.
- Stacking these constructs, we obtained functionally graded scaffolds.
- Gradient of mechanical properties, and anisotropic behaviour as in native tissue.