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Design of 3D Hybrid Composite Scaffolds: Effect of Composition on Scaffold Structure and Cell Proliferation

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Summary: The aim of this study was to develop multi-phase composite 3D scaffolds and to investigate the *in vitro* degradation performance, the cell seeding capacity and cells proliferation onto/into the composite scaffolds. Multi-phase composite scaffolds were formulated by solvent casting and particulate leaching technique, hydroxyapatite (HAP) was used to improve the mechanical properties of poly[(DL-lactide)-coglycolide] (PLGA) polymer scaffold, while chitosan (CTS) was added to the formulation for its bioactivity, osteoconductivity and bioadhesive properties. The *in vitro* degradation results demonstrated that the composite scaffold had a degradation rate correlating with their composition and structural features. Adult fibroblast cells were seeded on the surface of the composite scaffolds. Cell viability and long-term proliferation onto/into composite scaffolds were evaluated. The results showed that viable cells attached on the surface of scaffolds gradually migrated into the porous scaffold. These results suggest that the two-phase scaffold, namely PLGA/HAP and PLGA/CTS composite scaffolds, are promising grafts for bone tissue engineering. Further studies are in progress to design an improved three-phase composite construct obtained combining PLGA, HAP and CTS in a wafer-like scaffold.

Keywords: 3D scaffolds; cell proliferation; composite; *in vitro* degradation

Introduction

Bone and joint substitutes are commonly made of metals, ceramics, polymers and their composites. In general, metals and ceramics are used in hard tissue regeneration, while polymers are applied for soft tissue due to their limited mechanical properties.[1]

The fundamental requirements of the biomaterials intended for bone tissue engineering are biocompatible surface and suitable mechanical properties. Conventional single-phase materials can not satisfy the requirements for tissue engineering applications because they do not provide the structural and mechanical features required for bone grafting. Moreover, their characteristics are highly different compared with the autologous bone matrix.[2,3]

The mineral phase in the form of granules, powder or porous block can be combined to the polymer phase and formulated as porous scaffold. Using a multi-phase matrix, it is possible to benefit of the polymer biodegradability and the bioactivity of inorganic components. Moreover, the mechanical properties of the composite matrix can be manipulated making them comparable to the features of bone tissue.[4–6]

According to the capability to get interactions with the host tissue, the composite bone grafts can be classified into

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three types: i) nearly bioinert, ii) bioactive and iii) biodegradable. For bioinert graft, the interface of the matrix is neither chemically nor biologically bonded to the surrounding tissue, while interfacial interactions between the composite graft and the living tissue are achieved in the case of bioactive graft. The biodegradable type is designed to biodegrade over the time and gradually be replaced by the new bone tissue.[7]

PLGA is a synthetic polymer being approved by the FDA to be widely used as implantable screws, pins, drug delivery devices, and tissue engineering scaffolds because of its biocompatibility, biodegradability, and processability. Moreover, the PLGA physico-chemical features, as degradation rate and mechanical properties, can be controlled by varying its composition and molecular weight.[8]

HAP is part of the calcium phosphate-based bioceramics family. Due to its the atoxicity, the bioactivity, the osteoconductivity, and the similarity of the its chemical and crystalline structures to the natural bone mineral, HAP is widely used for bone repair.

Moreover, nano-sized HAP can improve the mechanical properties and support calcium and phosphate delivery when it is in contact with physiological fluids. Therefore, incorporation of the nano-HAP into a polymer matrix is assumed to mimic the natural bone structure as well as enhance cell growth and response.[9,10]

CTS is a polysaccharide copolymer consisting of alternating β-(1 → 4)-2-acetamido-d-glucose and β-(1 → 4)-2-amino-D-glucose unit linkages. Over the past two decades, CTS has been developed considerably in biomedical applications due to its high biocompatibility, biodegradability, bioadhesion properties, suitability for cell in growth, osteoconduction and intrinsic antibacterial nature. CTS offers a wide range of applications, including cartilage tissue engineering, wound healing and orthopedic applications.[11–13]

The aim of this research project is to develop a three-phase composite scaffold intended for bone tissue regeneration, combining the biodegradability of the synthetic hydrophobic polymer (PLGA) with the bioactivity and osteoconductivity of the inorganic phase (hydroxyapaptite nano-powder, HAP) and/or a secondary polymeric phase of natural origin and hydrophilic, as chitosan. To the best of our knowledge no research has been performed so far on three-phase composite scaffolds based on PLGA/HA/CTS for bone tissue regeneration. The present research study represent certainly an advancement in design and development of multi-phase composite scaffolds intended bone tissue regeneration.

Material and Methods

Poly[(DL-lactide)-coglycolide] polymer (PLGA 8515 DLG 7E, M_w 120 kDa, M_n 97 kDa) was purchased from Lakeshore Biomaterials, Birmingham (USA), Chitosan chloride (CTS, Protosan CL213, M_w 300,000–350,000 Da, deacetylation degree 82%, hydrochloric acid content 10–20%) was obtained from Pronova Biomedical, Norway and HAP particle size < 200 nm was purchased from Sigma Aldrich, Milan (Italy). 1,4-Dioxane, used for scaffolds preparation, was obtained from Carlo Erba, Milan (Italy). The water used in the preparation of scaffolds was distilled and filtered through 0.22 μm Millipore membrane filters (Millipore Corporation, Massachusetts, USA). Unless specified, all other solvents and reagents were of analytical grade.

Preparation of 3D Scaffolds

Scaffolds were prepared by solvent/casting particulate leaching method using 1,4-Dioxane as solvent.[14] Porogen paraffin particles were sieved onto a 600–1180 μm diameter sieves; 700 mg of sieved porogen particles (70% w/w) were placed into each Teflon mould.

700 μl of PLGA solution (15% w/v in 1,4-Dioxane) were cast drop by drop into the Teflon moulds filled with porogen. The

moulds containing the porogen and the polymer solution were first maintained at room temperature (RT), overnight to permit the diffusion of the polymer solution through the porogen particles and then they were placed at −25 °C for 24 hours. The frozen porogen/polymer mixtures were freeze-dried at −50 °C for 12 hours to completely remove the solvent. The scaffolds were dialyzed in hexane and cyclohexane at RT for 3 days to remove porogen particles. After dialysis the scaffolds were freeze-dried at −50 °C overnight. The prepared scaffolds were stored in a desiccators at −25 °C until characterization.

Hybrid composite scaffolds were formulated adding the HAP powder (PLGA: HAP ratio, 1:1) and the CTS solution into the PLGA polymer solution, the homogeneous suspensions were dropped into the Teflon mould following the protocol set up for PLGA scaffolds. The composition scaffolds is summarized in Table 1.

Characterization of PLGA Scaffolds

The architecture of scaffolds was examined by scanning electron microscopy (SEM) (Jeol, Cx, Temcam, Jed, Tokyo; Japan). Samples were sputtered with an Au/Pd coating in argon atmosphere. The coating was repeated twice to allow complete coating of the trabecular structure.

The density and porosity values of the scaffolds were measured by a modified liquid displacement method.[14] Ethanol was chosen as the displacement liquid because it penetrated easily into the pores, it is a non-solvent of the used polymers (PLGA and CTS) and the HAP powder, therefore it did not induce polymer matrix shrinkage or swelling. A weighted scaffold (W) was immersed in a graduated cylinder containing a known volume (V_1) of ethanol. The sample was kept in the non-solvent for 10 min, and then a set of evacuation-repressurization cycles was conducted to force the ethanol into the pore structure. Cycling was continued until no air bubbles were observed from the surface scaffold. The total volume of the ethanol and ethanol-soaked scaffold was then recorded as V_2. The volume difference, (V_2-V_1), was the volume of the scaffold skeleton. The ethanol-soaked scaffold was then removed from the cylinder and the residual ethanol volume was recorded as V_3. The volume (V_1-V_3), that is the ethanol volume retained in the porous scaffold, was defined as the pore volume of the scaffold. The total volume of the scaffold was calculated as follows:

$$V = (V_2 - V_1) + (V_1 - V_3) = V_2 - V_3$$

The density of the scaffold (d) was expressed as:

$$d = W/(V_2 - V_3)$$

And the porosity of the scaffold (ε) expressed as percentage (%) was calculated by:

$$\varepsilon(\%) = (V_1 - V_3)/(V_2 - V_3)^{*}100$$

The density and porosity were determined in triplicate (n = 3) and expressed as mean ± standard deviation, Table 1.

Glass Transition Temperature Determination

Glass transition temperature (T_g) was determined by means of a 2910 modulated differential scanning calorimeter, MDSC (TA Instruments, Delaware, USA), fitted with a standard DSC cell, and equipped with a liquid nitrogen cooling accessory (LNCA). Samples of about 10 mg were

Table 1.
Composition, PLGA M_w, porosity, pore size and apparent density of 3D scaffolds.

Scaffold #	PLGA % (w/v)	CTS % (w/v)	HAP % (%)	PLGA M_w (kDa)	Porosity (%)	Pore size μm	Density (g/mL)
1	15	–	–	106	72.08 ± 0.9	125–400	0.159 ± 0.03
2	15	–	15	85	84.1 ± 1.4	400–700	0.24 ± 0.05
3	15	2	–	105	83.12 ± 1.95	200–400	0.17 ± 0.013
4	15	2	15	83	nd	nd	–

quantitatively transferred to hermetically sealed aluminium pans and subjected to two cooling and heating cycles from −60 °C to +60 °C with cooling and heating rates of 5 °C/min. The DSC cell was purged with dry nitrogen at 40 mL/min.

The baseline correction was performed by recording a run with empty pans. The system was calibrated both in temperature and enthalpy with indium as standard material. The data were processed with Thermal Solutions software (TA Instruments, USA) and the results were expressed as the mean of three determinations.

Mechanical Properties

The compression and tension mechanical properties of scaffolds including compressive strength and compressive modulus were measured with an electromagnetic testing machine (Enduratec Elf 3200, Bose Corporation, Eden Prairie, MN, USA). The machine was equipped with a load cell of 220N. Different grips were used with the machine depending on the test configuration, i.e. compression or tension. Compression tests were done under displacement control, at a velocity of 0.1 mm/s till to reach the 40% of initial height. Tension tests were done under displacement control, at a velocity of 0.1 mm/s until the scaffold failure was achieved. The stress–strain curves were plotted and used to calculate the compressive modulus (Ec) and yield strength (Et). The compressive modulus and compressive strength were calculated as the average of five measurements per scaffold and expressed as mean ± standard deviation (SD).

In Vitro Biological Studies

Cell Culture and 3D Scaffold Seeding

Adult dermal fibroblast as primary cells were purchased from International PBI (Milan, Italy). The cells were cultured in DMEM containing 10% foetal bovine serum (FBS, Eu approved) and 1% antibiotic solution (100 U/ml penicillin, 100 ug/ml streptomycin, Sigma-Aldrich). After expansion, the cells were detached for the cell seeding experiments. A cell seeding of 5×10^5 cells/scaffold was used for all 3D scaffolds, all samples were sanitized by washing with ethanol (70% v/v) for three times followed by rinsing and subsequent conditioning with sterile physiological solution (0.9% w/v), prior to the cell culture.

For the surface seeding, a concentrated cell suspension (50 μl) containing complete DMEM was dropped on the top of each scaffold. The scaffolds were incubated for 3 h to allow cell attachment. At the end of the incubation, the scaffolds were submersed into complete DMEM.

Cells seeded on the scaffolds were determined after 3h of incubation using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium) assay.[15] Cells in 2D culture (bottom of culture plate) seeded with equivalent numbers were used as total cell number controls (CRT). After 3h, the cell-seeded scaffolds and controls (cells seeded on culture plate) were thoroughly rinsed in PBS and then 300 μL of MTT solution (5 mg/mL in DMEM without serum) were added into wells. The scaffolds were submersed with fresh in DMEM without serum and incubated for 2.5 h at 37 °C to allow MTT reduction by mitochondrial dehydrogenase in viable cells. Afterwards, a suitable detergent was added to dissolve the resulting blue formazane crystals. Results were revealed by a multiwell scanning spectrophotometer (Microplate Reader Model 680, Bio-Rad Laboratories, USA). The optical density (OD) was measured at 595 nm with 655 nm as reference wavelength. The results were expressed as cell seeding capacity (%) which were determined as follow:

$$\text{cell seeding capacity (\%)} = \frac{\text{number of cells attached on scaffold surface}}{\text{number of seeded cells } (5 \times 10^5 \text{ cells/scaffold})} \bullet 100$$

Cell Proliferation Study

Cell proliferation studies were assessed on 3D-scaffolds (Sc #1, #2 and #3). All samples were sanitized by washing with ethanol (70% v/v) for three times followed by rinsing and conditioning with sterile physiological solution (0.9% w/v) prior to cell

culture. Fibroblast cells were seeded on the sanitized samples at fixed cell concentration (5×10^5 cells/scaffold) following the cell-seeding protocol. The samples were kept at 37 °C in an atmosphere of 5% CO_2 for an incubation time up to 21 days. At scheduled times (7, 10, 14, and 21 days) the scaffolds were removed from their respective wells and placed in new wells, after each time point, in order to ensure that only cells attached to the test samples were considered for MTT analysis. Control cultures were grown on the bottom of wells.

In Vitro Degradation Studies

The scaffolds were incubated at 37 °C in 10 ml of phosphate buffer saline (PBS), pH 7.4. The incubation buffer from each test tube was withdrawn, collected and replaced with fresh PBS buffer at regular intervals (twice a week).

At scheduled times (3, 7, 10, 14, 21 and 28 days) samples were recovered, washed with distilled water and lyophilized (Lio 5P, Cinquepascal s.r.l., Milan, Italy) at about −50 °C, for 24 h. The degradation study was performed on scaffolds Sc #1, #2 and #3. The *in vitro* study included the determination of the PLGA M_w variation, the weight loss, the water uptake and the measurement of buffer pH during the *in vitro* degradation study. The data are expressed as average of three parallel samples.

Water Uptake Determination

The amount of water absorbed (WA) in the scaffold incubated at 37 °C in PBS was determined gravimetrically (Mettler Toledo AG 245, Milan, Italy). At scheduled times, the scaffolds were weighed immediately after recovering and subsequently rinsed with distilled water. The water content was computed as follows (1):

$$\text{Water Content}\,(\%) = \frac{W_t - W_o}{W_t} \bullet 100 \qquad (1)$$

where W_t is the weight of wet scaffold at time t, and W_0 is the initial weight of the dry scaffold. Water uptake was determined in triplicate (n = 3).

Gel Permeation Chromatography

Gel Permeation Chromatography (GPC) was used to evaluate the weight-average molecular weight (M_w) of PLGA matrix during incubation in PBS (pH 7.4), at 37 °C.

The GPC apparatus consisted of three Ultrastyragel columns connected in series (7.7×250 mm each, with different pore diameters: 10^4 Å, 10^3 Å and 500 Å), a pump (Varian 9010, Milan, Italy), a Prostar 355 RI detector (Varian Milan, Italy), and software for computing M_w distribution (Galaxie Ws, ver. 1.8 Single-Instrument, Varian Milan, Italy). Raw polymer and scaffolds were dissolved in tetrahydrofuran (THF) at a concentration of 1–2 mg/mL, the sample solutions were filtered through a 0.45 μm filter (Millipore, Massachusset, USA) before injection into the GPC system, and they were eluted with THF at 1 mL/min flow rate. The weight-average molecular weight (M_w) of each sample was calculated using monodisperse polystyrene standards (M_w 1,000–150,000 Da).

Weight Loss Determination

The weight loss (WL) of prototypes was determined gravimetrically (Mettler Toledo AG 245, Milan, Italy) at scheduled times, the samples subjected to the incubation in PBS were freeze-dried before gravimetric analysis. The weight loss was determined in triplicate, and computed as follows (2):

$$\text{Weight Loss}\,(\%) = \frac{W'_0 - W'_t}{W'_o}\,100 \qquad (2)$$

where W'_0 is the weight of scaffolds before incubation, and W'_t the weight of the scaffolds after incubation and freeze drying (n = 3).

pH of Degradation Buffer

The pH of the buffer along the *in vitro* degradation study was measured with a pHmeter (pHmeter 827 pHLab; Metrohm, Switzerland) twice a week.

Results and Discussion

Scaffold Characterization

Figure 1 shows the morphology of 3D scaffolds prepared by solvent casting particulate leaching technique using paraffin spheres as porogen.[14] The paraffin spheres were removed by dialysis using hexane and cyclohexane as extraction solvents, the selection of these solvents was performed considering the paraffin solubility, their melting temperature and the concentration limits reported in ICH Q3C (R3).

Hexan is a good solvent for paraffin, nevertheless its melting point is lower (−96 °C) than the freezing temperature (−25 °C) used to prepare the samples for freeze-drying cycle. Cyclohexan has a melting point of 6.47 °C, thus it assures complete solvent sublimation by lyophilisation. Moreover, the ICH Q3 concentration limit of the cyclohexan is 3880 ppm, against 290 ppm of the hexan. The residual amount of solvents used for the preparation of prototypes was calculated by the gas chromatography, the residual concentrations resulted to be always below to the concentration limits (unreported data).

Table 1 reports GPC data referred to the freeze-dried 3D-scaffolds. The analyses were performed to assess how the preparation protocol affected the chemical composition of PLGA polymer. On the basis of GPC data, it is possible to identify the use of organic solvents as the most critical process point. Results demonstrated that the organic solvents led to important variations of chemical properties, resulting in the reduction of average M_w. Moreover, the M_w reduction resulted to be dependent on the scaffold composition, in particular to the presence of inorganic component (HAP) in the polymeric matrix. GPC values demonstrated a M_w percentage reduction ranging between 12.5% and 29.18% with respect to the average M_w of polymer in bulk (120 kDa).

The final formulations were however stable at the selected storage conditions. Indeed, after few months of storage at 5 °C ± 2 °C, the scaffolds average M_w did not change (unreported data).

SEM images illustrate scaffolds characterized by high porosity and well interconnected pore network (Figure 1). The structure clearly changed with the composition: PLGA scaffold had irregular pore range from 125 to 400 μm (Figure 1, 1a-1c), a bubble-like structure was detected by SEM analysis which can be attributed to the

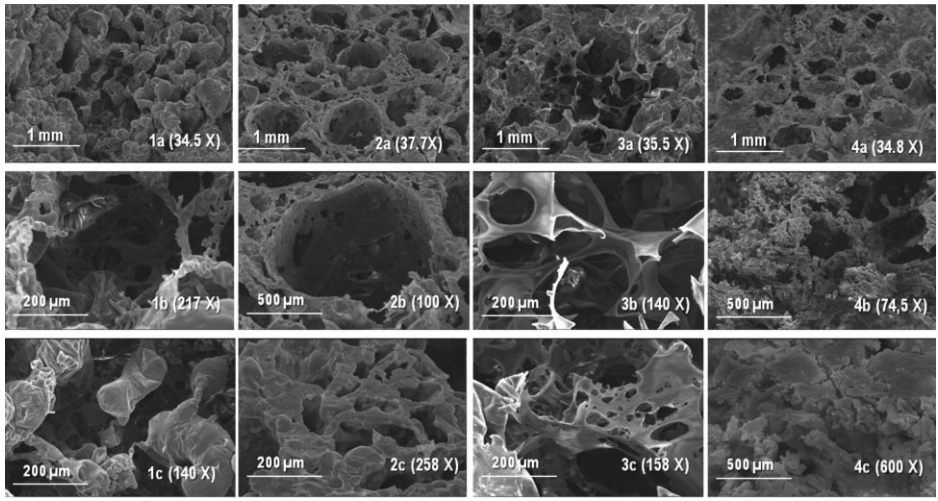

Figure 1.
Scanning electron micrographs of 3D scaffolds prepared using the solvent casting particulate leaching technique with paraffin spheres as porogen. a) view of transverse section at low magnification, b) interconnected porous network and c) wall pore structure.

distribution of the PLGA solution on the porogen spheres surface. The bubble-like structure was maintained both during the extraction of paraffin spheres and after the freeze-drying process.

The addition of HAP to the PLGA matrix led to a dense structure characterized by regular spherical pores, ranging between 400 and 700 μm (Figure 1, 2a-2c). The internal pore surface was rough and irregular for the presence of the HAP nano-powder. The microporosity may be rationalized considering the solid-liquid separation phenomena ascribed to crystallization of solvents, freezing time and temperature.

The double-phase composite scaffold obtained combining PLGA with CTS presents a highly porous structure characterized by well interconnected pores, micropores (50–100 μm) were detected on the surface of polymer fibers (Figure 1, 3a-3c). The microporosity may be rationalized considering the solid-liquid separation phenomena ascribed to crystallization of solvents, freezing time and temperature.

The three-phase composite scaffolds (Sc #4) has a low porosity, in particular the internal part of the construct showed a compact matrix with low macro- and microporosity. These findings were attributed to the precipitation of chitosan polymer when getting in contact with HAP leading to a insufficient porosity for the tissue engineering application (Figure 1, 4a-4c).

For all tested 3D scaffolds, the pore size measured by SEM analysis resulted to be lower compared with the original size of porogen spheres (600–1180 μm), which was considered a consequence of multiple factors: i) the polymer chains shrinkage during the freeze-drying cycles, and ii) the shape and size of solvent crystals formed during the freezing process.

The liquid displacement method confirmed the high porosity of scaffolds, which ranged from 72.08% to 84.1%, the apparent density changed with the polymer composition (Table 1). The highest apparent density was calculated for double-phase composite scaffold obtained combining the polymer with the inorganic component, HAP.

The compressive modulus and the compressive yield strength (Table 2) of double-phase scaffolds obtained with PLGA and HAP are significantly higher than the values calculated for the single-phase (Sc #1, PLGA) and the double-phase (Sc #3, PLGA/CTS) scaffolds. The data demonstrated the capability of HAP to improve the mechanical properties of the scaffold.

The DSC traces recorded on 3D scaffolds and PLGA polymer are reported in Table 2. Data were collected up to 200 °C since above this temperature incipient decomposition of polymer takes place. The baseline deflection corresponding to the glass transition and occurs at 43.32 °C for PLGA raw polymer: It is possible to note that T_g values recorded on 3D scaffolds are lower than that of PLGA polymer. Such an evidence highlights how the composition and eventually the structure of scaffold could affect the polymer thermal properties leading to lower glass transition temperatures.[16]

In Vitro Biological Studies

The cell seeding study demonstrated good cell seeding capacity with values ranging between 60 and 95% (Figure 2).

The incubation of scaffolds with cells demonstrated that the 3D scaffolds were

Table 2.
Compressive modulus, yield strength and T_g values of 3D scaffolds.

	Scaffold #			
	1	2	3	4
Compressive modulus (MPa)	1.7 ± 0.6	4.9 ± 0.4	3.27 ± 0.02	–
Yield strength (MPa)	0.5 ± 0.09	0.8 ± 0.1	0.11 ± 0.05	–
T_g (°C)	25.5	18.56	22	21

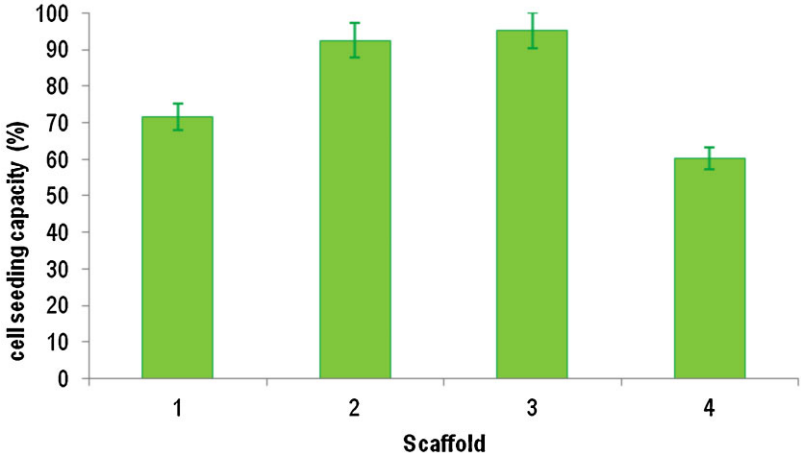

Figure 2.
Cell seeding capacity on 3D composite scaffolds. Cells (5×10^5/scaffold) were seeded on scaffold surface and then incubated in DMEM with FBS at 37 °C, 5% CO_2.

cytocompatible, and no toxic by-products (as degradation products or solvent residuals) were released from the samples. Cell seeding data resulted to be congruent with the structural features highlighted by SEM analyses and by the experiments carried out to measure pore size. The lowest number of viable cells was found out onto the prototypes with the smallest pore size (Sc # 1 and 4).

The cell proliferation was seen to increase in the double-phase composite scaffold from the 3rd to 14th day of incubation in DMEM at 37 °C (Figure 3). A reduced proliferation rate was calculated for the single-phase (Sc #1, PLGA) and the

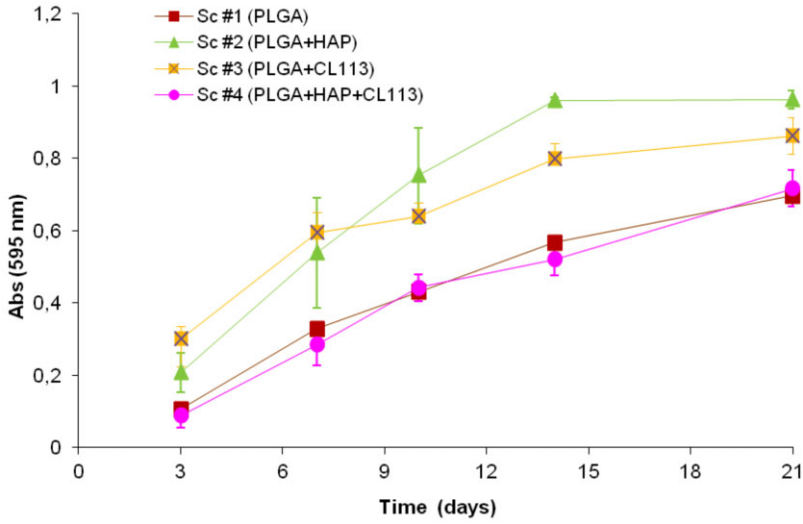

Figure 3.
Long-term cells proliferation study. Cells (5×10^5/scaffold) were seeded on the scaffold surface and incubated in DMEM with FBS at 37 °C, 5% CO_2 for 21 days. The equivalent cell number cultured on the well plates (2D cell cultured) was used as control.

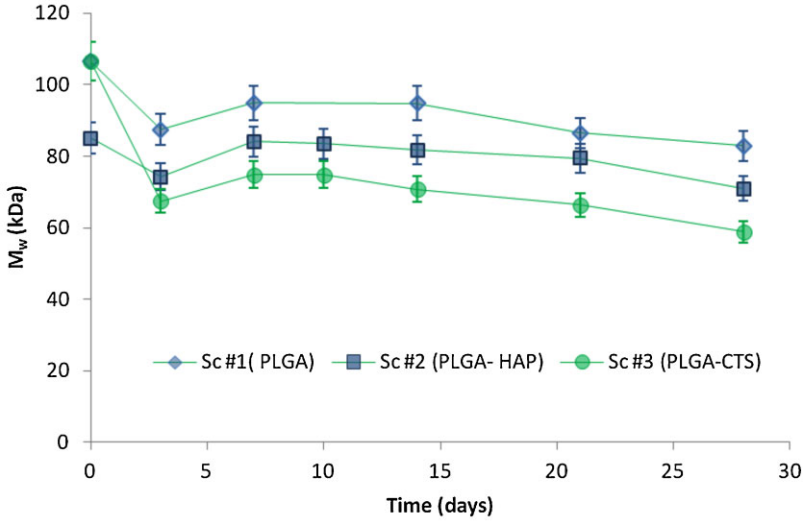

Figure 4.
Molecular weight variation profiles (M_w) of Sc #1, #2 and #3 incubated in PBS, pH 7.4 at 37 °C.

three-phase composite scaffolds (Sc #4, PLGA/HAP/CTS), till to reach the optical density of 0.6 after 21 days of incubation. These data could be explained taking in consideration: i) the higher porosity of Sc # 2 and #3, which usually means a higher surface area/volume ratio, and thus promotes cell adhesion to the scaffold and cell culture proliferation and ii) the composition of composite scaffolds, the inorganic component (HAP) and the hydrophilic natural polymer (CTS) are defined as bioactive compounds.

In Vitro Degradation Studies

The *in vitro* degradation study was assessed on the single- and the double-phase scaffolds (Sc #1, #2 and #3) and it was set-up following the approach used to make *the vitro* proliferation study. 3D scaffolds were

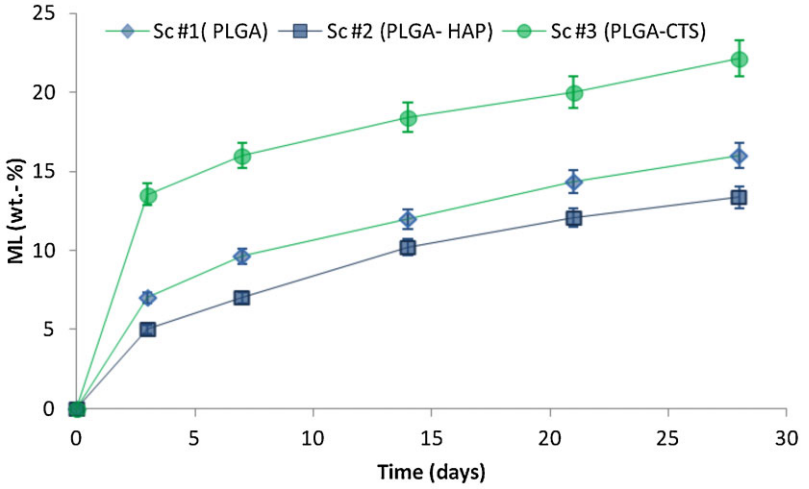

Figure 5.
Mass loss (wt.-%) of Sc #1, 2 and 3 incubated in PBS, pH 7.4 at 37 °C. Incubation time: 28 days.

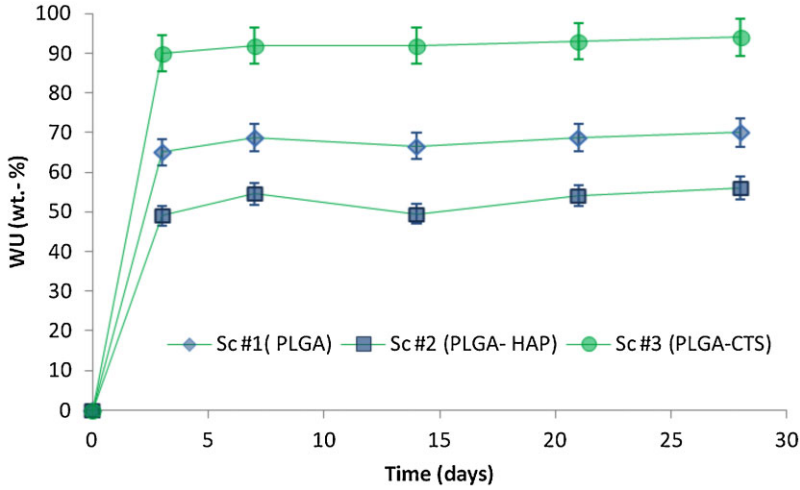

Figure 6.
Water uptake (wt.-%) of Sc #1, #2 and #3 incubated in PBS, pH 7.4 at 37 °C. Incubation time: 28 days.

incubated in KRH at 37 °C, for 35 days in static conditions. Data demonstrated that the degradation rate of Sc #3 was much higher with respect to the Sc# 1 and #2, Figure 4. Sc #1 and 3 profiles presented a rapid reduction of M_w in the first 3 days till to reach a percentage reduction of 24.6 and 46.5% at day 28th, respectively.

The M_w reduction of Sc #2 ranged between 1 and 17.45% during all the incubation time.

GPC data were supported by mass loss experiments (Figure 5). The mass loss profiles of 3D prototypes presented similar trend and the mass loss rates were correlated to the M_w reduction and the water uptake profiles.

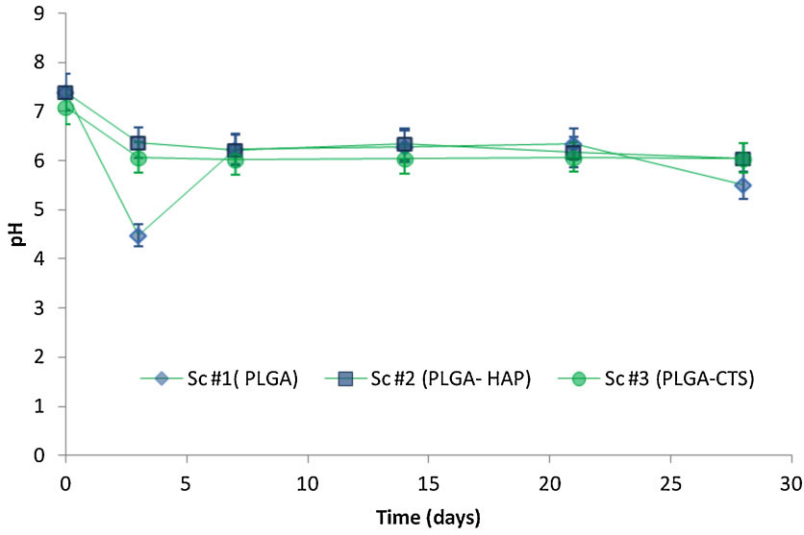

Figure 7.
pH shift of buffer during the in vitro degradation study of Sc #1, #2 and #3 incubated in PBS, pH 7.4 at 37 °C.

The water up take (WU) profiles are shown in Figure 6. As expected, the trabecular structure and the internal pore volume of 3D-scaffold allowed the entrapment of high amounts of water.

KRH pH were measured by pHmeter for all tested samples in order to complete and support the *in vitro* degradation experiments (Figure 7). The release of the acidic degradation products from the polymeric matrix induced the shift of pH to acidic values, in particular for the scaffold based on PLGA. The presence of HAP or CTS in the PLGA matrix buffered the acidic pH due to the release of acidic products.

Conclusion

The study performed on the composite scaffolds demonstrated the capability of using the composite approach to improve the mechanical properties of PLGA scaffold and to obtain a bioactive surface where the cells can attach and proliferate. The most promising results were obtained with double-phase composite scaffolds combining PLGA with HAP or CTS. Further studies are needed to optimize the design and the preparation procedure of the three-phase composite scaffolds, composed by the biodegradable PLGA polymer, bioactive HAP and CTS.

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