MESENCHYMAL STEM CELL BEHAVIOR ON BIOACTIVE POLY HYDROXYBUTYRATE/HYDROXYAPATITE NANOCOMPOSITE SCAFFOLD

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Tissue engineering is the use of a combination of cells, *engineering* and materials methods in order to improve or replace biological functions. In This study, Poly3-hydroxybutyrate\Hydroxyapatite nano composite (P3HB/nHA) scaffolds fabricated using solvent casting-salt leaching technique. *Tissue engineering* involves the use of a scaffold for the formation of new viable *tissue* for a medical purpose. Hydroxyapatite nanoparticles Synthesized via sol-gel method and then were confirmed by X-ray diffraction analysis and transmission electron microscopy. poly(3-hydroxybutyrate) was reinforced with different amount of nHA (0-10 wt%). Nanocomposite Scaffolds were characterized by Fourier transform infrared spectroscopy. Attachment and proliferation of mesenchymal stem cells were studied by Microculture Tetrazolium *Assay* (MTT) and Scaning electron microscopy. X-ray diffraction analysis and transmission electron microscopy confirmed that nanohydroxyapatite particles was synthesized successfully. Fourier transform infrared spectroscopy (FTIR) analysis showed that P3HB and HA coexist in the nano composite. According to SEM results, the pore size of the scaffold was about 200-250 μm. Cell culture studies demonstrated that addition of nHA to P3HB matrix improved proliferation of mesenchymal stem cells. This study could suggest the scaffolds with 10 wt%HA and 90% wt. NaCl as a better candidate for bone tissue engineering.

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1. Introduction

Tissue engineering is the method developed for the repairing of the damaged human tissues by the process of regeneration of living tissue. Generally, a scaffold is employed for this process and it is necessary that it must have properties which are essential for tissue engineering such as proper surface, architecture, mechanical and degradation properties. These properties should match with ones which are desirable for bone tissue engineering process [1-2]. The most commonly employed scaffolds for tissue engineering are synthetic biodegradable polymer matrix composites which contain bioactive ceramic phases. It's use is widely acceptable because of its biological, physical and mechanical ideality to the desirable features [3].

The most widely used synthetic materials for tissues engineering purposes are derived from Poly hydorxy alkanoates (PHAs) which belong to the biopolymer family [4].

PHAs are biodegradable polymers that have been used for biomedical applications such as tissue regeneration, drug delivery and patches [5-6]. Poly 3-hydroxybutyrate (P(3HB)) is a well-known member of PHA family which has extended degradation time and it is obtained mainly from microbial sources [6-7]. Other hallmarks of P (3HB) are its compatibility with a number of cell lines and piezoelectric properties which permit bone growth and regeneration [8-9].

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Wide variety composites of P (3HB) and bioactive inorganic phases, such as hydroxyapatite (HA) and Bioglass (BG) have been prepared by different researchers to improve strength and bioactivity of the composites, as reviewed elsewhere. Micro or nano particles may be added to the polymer matrix. However, in the case of PHA composites, mainly micro particles have been investigated previously[4]. But, due to the small size and huge specific surface area (specific properties of nano particles) of nano-sized HA, P(3HB) composites with this particles may have unique mechanical and biological properties [10-11].

In this study, poly 3-Hydroxy Butyrate, was used as the matrix of scaffold. The PHB/nHA composite scaffold was prepared using solvent casting- salt leaching technique. Great effort was devoted to study the effects of the amount of nano filler and spacer agent (NaCl partiles) on the cell bioactivity behavior of the nHA reinforced P(3HB) composite scaffold. Characterization techniques, such as SEM and FTIR, were used for cell bioactivity evaluation of the nano composite scaffolds.

2. Materials and methods

2.1. Materials

Calcium phosphate tetrahydrate $Ca(NO_3)_2.4H_2O$, Three ethyl phosphite, $(C_2H_5O)_3P$, Sodium Chloride, NaCl, and Ethanol were provided by Merk Co. NaCl crystal particles with size of 212-250 μm were sieved. Polyhydroxy butyrate (PHB) was purchased from Sigma Aldrich Chemical Co. (USA, CAS NUMBER: 26063-00-3, LOT NUMBER: S68924-099). Chloroform was supplied from Scharlau Co. (Spain) by 99.5% purity.

2.2. Scaffold Preparation

Nano hydroxyapatite particles synthesized as mentioned in the previous work [12]. In order to synthesize of scaffold, technique of salt leaching and solvent casting approach was employed. Agglomeration of the particles were prevented by ultrasonication stirring in acetone [12-13].Combination of ultrasonication and solution casting method was used for proper dispersion of nanoparticles in the polymer matrix. Briefly, 0.4 g PHB powder was dissolved in 10ml chloroform at 50±2°C to form 4 wt/v% polymer solution, followed by addition of 0, 2.5, 5, 7.5 and 10 wt% of nHA powders. After 30 min sonication (25 kHz, 340W), NaCl was added to mixture and stirred for 15 min. The size of salt particles was in the range of about 212-250 μm and the NaCl: P3HB ratio were selected at 70:30, 80:20 and 90:10. Subsequently, the mixture was instantly poured in petri-plates. After 48 hrs room drying, samples were dried in oven under vacuum for 24 hr. For salt leaching, the samples were immersed for 5 days in large amount of deionized water in orbital shaker and then dried in vacuum oven and room temperature for two days and one day respectively.

2.3. XRD and FTIR analysis

X-ray diffraction analysis (XRD) was performed using a Philips (X'PERT) XRD with Cu-Kα radiation, λ=1.54 Å, (Netherland) over the 2θ range of 10- 80° for nHA powder, pure PHB and PHB/nHA composite scaffold. The crystallite sizes of the powders in nanometers were estimated using the Debye- Scherrer equation as shown below:

$$t = \frac{0.9\lambda}{B\cos\theta} \quad (1)$$

where λ is the wavelength of CuKα (0.154 nm), B is the peak width at half-maximum intensity of HA in radians and θ is the angle of the latter peak in degrees.

Fourier transform infrared absorption spectra (FTIR) of the polymer, composite scaffolds and nHA powders were measured in the spectral range of 400–4000 cm^{-1} using Jasco FTIR 680 plus-Japan.

2.4. Microscopic observation

Transmission electron microscopy (TEM) was used to investigate the morphology and size of the synthesized nHA. The microstructure of composite scaffolds was observed by scanning electron microscopy (SEM, SERON TECH. AIS2100, South Korea). Prior to examination, each sample was coated with gold. Energy Dispersive X-ray Analysis (EDXA, map of Ca) was also used to observe the distribution of nHA in the polymer matrix.

2.5. *In vitro* cytocompatibility evaluation

Prior to cell culture initiation, Disks with a 16 mm diameter were cut from the scaffolds and were sterilized by ethanol (70%). Then, 10000 passaged mesenchymal stem cells (MScs) were suspended in 500 µl Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin, and streptomycin (all from Invitrogen), and were placed on the surfaces of the scaffold disks located in wells of 24-well culture plates. The cultures were incubated in the atmosphere of 5% CO_2 at 37 °C.

Surface of scaffolds was studied by SEM to observe attachment and spreading (proliferation) of cells. After two weeks incubation, Specimens were fixed in 2.5% glutaraldehyde and then dehydrated by 50, 70, 90 and 100% ethyl alcohols. After drying, Samples were attached to stubs and gold coated in order to be examined by SEM.

The proliferation of MSCs on scaffolds was evaluated via MTT assay which determines the concentration of living cells. MTT (Sigma, USA) was dissolved in phosphate buffered saline (PBS) (5 mg/ml) and was then sterilized by filtration. Sterilized Scaffolds were placed in a 24-well culture plate and seeded with a cell density of 10000 cells/cm^2. After 1and 7 days of cell seeding, 50 µl of MTT solution was added to each well (n= 3) to extract the formazan crystals by slow shaking. After that, 100 µl of dimethyl sulfoxide (Sigma, USA) was added to each well.

Absorbance of samples was measured at 570 nm wavelength using micro plate reader. The same procedure was performed for cultured cells in tissue culture polystyrene (TCPS) as control [14].

2.6. Statistical analysis

Data are reported as means ± standard deviation of mean. One-way ANOVA was used for Statistical analysis. P-values indicated statistical significance at $p < 0.05$.

3. Results and discussions

3.1. Nano HA

Fig 1 shows XRD pattern and TEM image of nHA. There are three sharp peaks at approximately 32.2°, 32.6° and 33.2° which are the main peaks if crystalline hydroxyapatite. Fig 1b shows that the size of nHA particles is about 30-40 nm. The crystallite size of nHA was also calculated by Debye- Scherrer equation. The size of crystallites was about 35 nm which was in accordance to particle size shown in Fig 1b. It can be seen from Fig 1b that high free surface of nanoparticles causes some agglomeration.

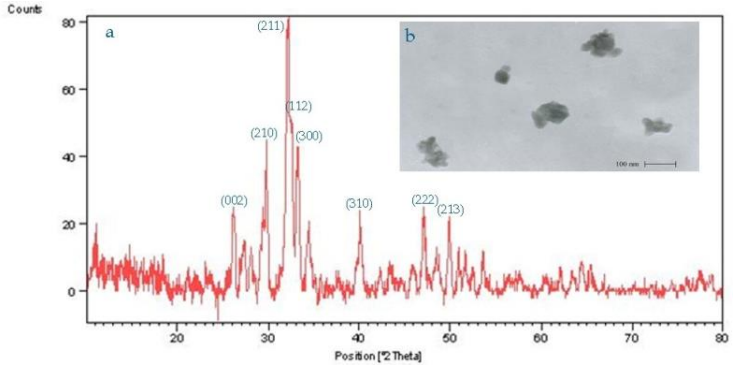

Fig 1.(a) X-ray diffraction pattern, (b) TEM micrograph of the synthesized nHA.

3.2. Microstructure and elemental X-RAY MAP

The microstructure of scaffolds prepared with different amount of NaCl is shown in Fig 2. SEM images, demonstrated uniform pore size of 200–250 µm which is suitable for osteoblast migration [14-15]. The closed pores of the prepared Scaffolds were decreased with increasing of NaCl. The prepared scaffold with 90 wt% NaCl in term of open pores has the best structure. It can be seen from Fig 2c the more interconnectivity in prepared scaffold with 90 wt% NaCl, because of the open pores, which is essential for cell migration, waste removal and nutrient supply to the scaffold in bone tissue engineering [16]. The figure is also showing no negative effect of nano particles on pore structure.

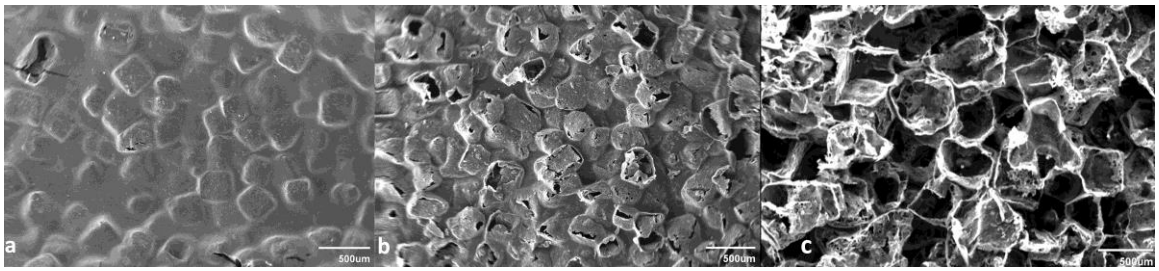

Fig 2. SEM micrograph of scaffolds prepared with (a) 70 wt% NaCl, (b) 80 wt% NaCl and (c) 90 wt% NaCl.

3.3. FTIR analysis

FTIR analysis of nHA, PHB and PHB/nHA composite scaffold is depicted in **Fig 3.** Peaks in range of 570-629 Cm^{-1} is belonged to bending or deformation vibration mode of O–P–O bonds in PO_4^{3-}groups of Hydroxyapatite (17) and peak in 1025 Cm^{-1} is belonged to stretching vibration mode of PO_4^{3-} ion of nHA (11).

The peaks at 3437 and 1637 cm^{-1} are relevant to the bending modes of hydroxyl group in the adsorbed water, while the peaks at 3753 and 633 cm^{-1} are assigned to the stretching vibration of the hydroxyl group in crystal structure of hydroxyapatite [11,17-18].

Peaks in 1055, 1128 and 1176 Cm^{-1} are belonged to C-O bond in PHB. Peak in 1375Cm^{-1} is belonged to Stretching vibration mode of CH_3 of PHB. Stretching vibration of carbonyl (C=O) is seen in 1724 Cm^{-1}. Peak of CH in PHB is seen at 2900Cm^{-1}. PHB also has OH group that it's peak is seen in 3400-3550 Cm^{-1}.

As shown in Fig 3, the intensity of C=O absorption peaks of PHB decreases obviously in the nano composite. The C=O vibration peak of pure PHB at 1724 also shifts to 1721 cm^{-1} in the nano composite. This is probably due to hydrogen bonding between carbonyl group (C=O) in PHB and some susceptible functional groups in nHA. Comparing PO_4^{3-} peaks in bending and stretching modes shows reduction intensity of mentioned peaks in nano composite than nHA. Wave number of PO_4^{3-} peak is also reduced to 566, 596 and 620 Cm^{-1} in nano composite than nHA. It is probably due to hydrogen bonding between PO_4^{3-} and terminated hydroxyl group of PHB.

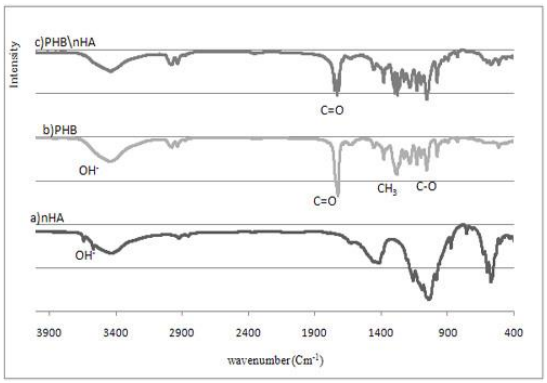

Fig 3. FTIR spectrum of (a) nHA, (b) PHB and (c) PHB/nHA composite scaffold

3.4. Proliferation of mesenchymal stem cells (MScs)

In this study, biocompatibility of scaffolds was determined by MTT assay which revealed the proliferation rate and viability of MSCs on pure PHB and nano composite scaffolds (Fig 4). To investigate the mitochondrial functions of cultured cells, the reduction of MTT reagent was assessed as an assay of mitochondrial redox activity. MTT reagent is a pale yellow substance that can be reduced to a dark blue formazan product by mitochondrial succinate dehydrogenase when incubated with viable cells. Therefore, the production of formazan can reflect the level of cell viability on the material [19].

Fig 4 shows the absorbance of formazan produced by viable cells attached on the P3HB/nHA and pure P3HB scaffolds in comparison to the control sample (TCPs) after one day and one week culture. With an equal initial cell density on day 1, no significant difference was observed between the rate of proliferation on different scaffolds and TCPs . On day 7, the highest cellularity was measured on PHB/7.5 wt% nHA compared to other groups. Of note, the cells on the nano composite proliferated to significantly higher degrees than those on the pure P3HB scaffold ($p < 0.05$). Recently, it was reported that nano scale architecture in a three dimensional improved human serum protein adsorption [20-21]. Improved protein adsorption can enhance cell attachment to the substrate, and therefore, can enhance cell proliferation. Wang et al. showed that HA blending within a P3HB scaffold led to an increase of osteoblast responses, which may have been related to the surface morphology and to the exposed HA particles on the polymer surface (4). In another study, Guan et al., demonstrated that the incorporation of nHA particles in to chitosan nano fibrous scaffolds led to significant bone formation oriented outcomes compared to that of the pure electrospun chitosan scaffolds [22].

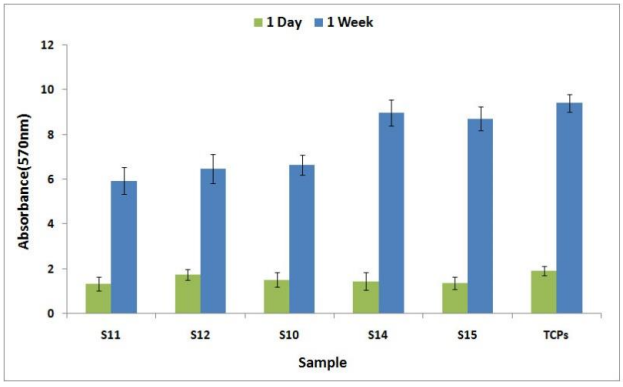

Fig 4. Proliferation of MSCs on scaffolds and TCPS during 1 day and 1 week culture time ($P < 0.05$).

3.5. Cell morphology

The nano composite scaffold (PHB/7.5 wt% nHA) and unfilled P(3HB) scaffold were examined under SEM to observe the MSCs spreading and attachment on the surface of the samples after 2 weeks culture. MSCs showed a spreading and typical morphology on PHB/nHA and pure PHB scaffold (Fig 5). There is a colorless layer of cells on the surface of scaffolds.

According to SEM results, cellular attachment and growth on nano composite scaffold is very better than that on P3HB scaffolds. It demonstrates the constructive effect of hydroxyapatite nano particles on cell growth and attachment.

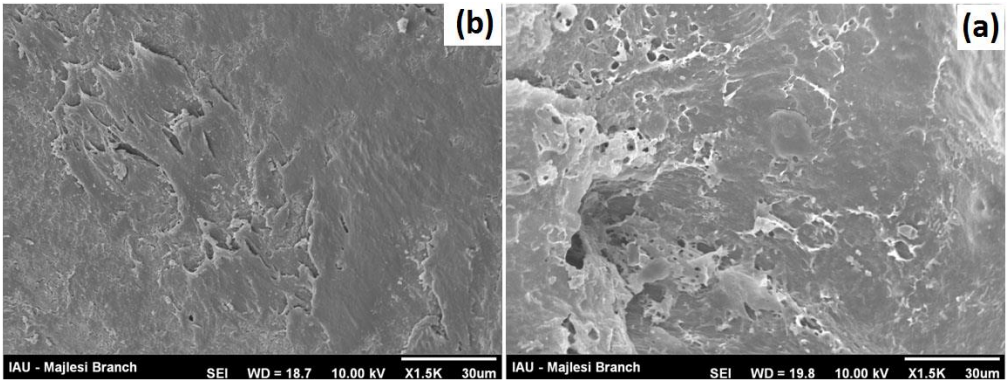

Fig 5. Morphology of MSCs on surface of (a) pure PHB scaffold and (b) nano composite scaffold containing 7.5 wt% nHA

4. Conclusion

In summary, pure P3HB and nano composite scaffolds were successfully fabricated using solvent casting/salt leaching technique. As well as it is mentioned that PHB/nHA composite scaffolds prepared with 90 wt% NaCl has the best microstructure. Based on the SEM results, it was found that P3HB/nHA nano composite scaffolds have a better adhesion tendency to MScs than P3HB scaffolds. It was also found that the proliferation of nano composite scaffolds was higher than that of P3HB scaffolds**.** Due to the enhanced cell proliferation in comparison with conventional composite scaffold, the PHB/nHA composite scaffold supports a more promising potential for the repair and replacement of bone. Finally, it is concluded that PHB/nHA composite scaffolds prepared with 90 wt% NaCl and 7.5 wt% nHA is the best candidate for bone tissue engineering.

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