Composite bioceramics/polymer electrospun scaffolds for regenerative medicine

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Abstract.
The main goal of this study was to succeed in the relevant association of well-known osteoconductive biphasic calcium phosphate (BCP) made of Hydroxyapatite (20% HA) and β-Tricalcium Phosphate (80% β-TCP) crystallographic phases and resorbable poly(L-lactide-co-D,L-lactide)(PLDLLA) 3D matrices synthesized by electrospinning. Two types of mineral particles were obtained, BCP new hollow granules, and classical BCP particles. It appeared that hollow shells/PLDLLA composite 3D matrices allowed higher cell adhesion *in vitro,* thanks to internal concavities and are promising scaffolds in terms of cell carrying.

Introduction

The need of both bioresorbable and osteoconductive 3D scaffolds able to recruit stem cells and enhance their differentiation to new bone, lead to innovative composite biomaterials synthesis for bone tissue engineering and gene therapy [1]. Electrospinning is a room temperature process invented in 1934 [2] involving nano or micro fibers-based scaffolds able to be used for bone ingrowth as literature mentioned it since the late 90s [3, 4]. Its biomimetic properties regarding extracellular matrix microstructure similarities enhanced cell attachment and adhesion [5-7]and justified this method to design fiber-based 3D scaffolds [7, 8]. One-step electrospinning process is then considered as a promising technology for regenerative medicine since it is low-cost, rapid and easy to perform [9]. Among all the available polymers in bone tissue engineering, such as polycaprolactone (PCL) or poly(lactic acid-co-glycol acid) (PLGA) [10, 11], poly(L-lactide-co-D,L-lactide)(PLDLLA) which is part of poly (α-hydroxyacids) family [12], was selected in our development according past studies results dealing with combination of BCP microparticles and PLDLLA for the elaboration of resorbable osteosynthesis with osteogenic properties [13-15].
As fiber -based scaffold need osteoconductive component to match bone tissue engineering specifications[16], addition of microparticles of hydroxyapatite (HA) or tricalcium phosphate (β-TCP) [17]to electrospun scaffolds have been shown to be relevant not only in term of cell adhesion enhancement, but also in cell differentiation involving extracellular matrix mineralization [18-20]. In this study biphasic calcium phosphate (BCP) new galenic formulation, that we called Hollow Shells, with a ratio HA/TCP of 20/80 were synthesized using specific granulation method so as to obtain a composite ceramic granule/3D fibers containing either macroscopic hollow shells or classical microscopic particles. Osteoconductive properties of these BCP biomaterials have been proven for decades [21, 22] and resorption-precipitation mechanism involved *in vivo* have been explained to be favorable to osteoinduction [23, 24].

Materials and Methods

Poly(L-lactide-co-D,L-lactide) was obtained from Biomatlante SA (Vigneux-de-Bretagne, France). After synthesis and sintering, hollow shells were passed through sieves in order to obtain granules (400 to 600 μm) and BCP microparticles of size less than 10 μm.

Thus two kinds of BCP were incorporated into the 3D scaffolds in two ways. In the first one, BCP microparticles were mixed with the polymer solution which was PLDLLA dissolved in choloroform at 4% w/v [25], and then projection was realized by electrospinning. The second one consisted on deposition on aluminium conductive support, of macroscopic granules called "hollow shells" being embedded during the electrospinning process.

The electrospinning device (Linarii Bioengineering, Italia) was set at 25kv, with a working distance of 20cm and a flow of 5cc/hour.

Three types of membranes were synthesized using PLDLLA:

    A. Polymer alone
    B. Composite Polymer/Bioceramic with macroscopic BCP hollow shells
    C. Composite Polymer/Bioceramic with microscopic classical BCP particles

Scanning Electron Microscopy (SEM) Fitted with Energy Dispersive X-Ray (EDX) were performed to localize BCP microparticles. Image analysis for fibers diameter was realized by manually measurement of pictures (n=5) for each kind of 3D scaffold. Density calculation were realized.

Cell adhesion assessment on membranes A, B and C was realized using MC3T3 osteoblastic murine cells after 3 days in contact of membranes in alpha MEM culture medium (10% Foetal bovine serum, 1% streptomycin/penicillin, 1% L-glutamin). Cells were fixed with paraformaldehyde (4% in phosphate buffer saline) then the immune-independent actin marker phalloidin (Sigma-Aldrich) have been incorporated to the seeded cells after using a permeabilization buffer (Tween 0.05%, BSA 3% in PBS). Nucleus staining was obtained thanks to Topro-3 DNA dye (Invitrogen). Stained cells were then observed *in situ* using fluorescent and confocal microscopy, in order to determine cells density and cells morphology.

Results

Hollow shells were observed after sintering and before electrospining process. A large macropore concavity (figure 1a) appeared in each granule with a diameter of between 300μm to 500μm and high microporosities content less than 1 μm (figure 1b).

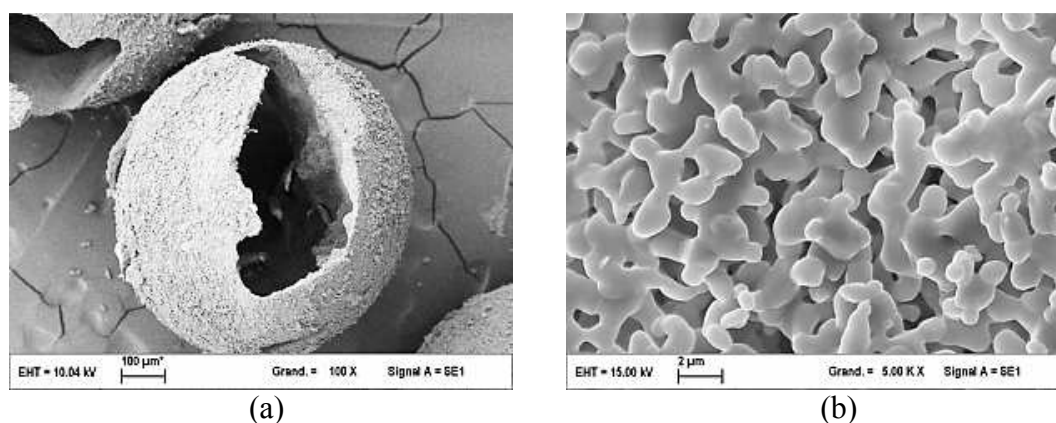(a)   (b)

Figure 1: Hollow Shells characterization before electrospinning process

Electrospun fibers of all membranes types (A, B, C) were found to have a bimodal distribution of their diameters, fiber diameters 4 μm (class I) and fiber diameters 1 μm (class II) as seen in figure 2. Hollow shells of type B scaffolds were embedded in lower density of fibers comparing to the rest of the scaffold (figure 2b), with a round area around hollow shells of approximately 500 μm in diameter.
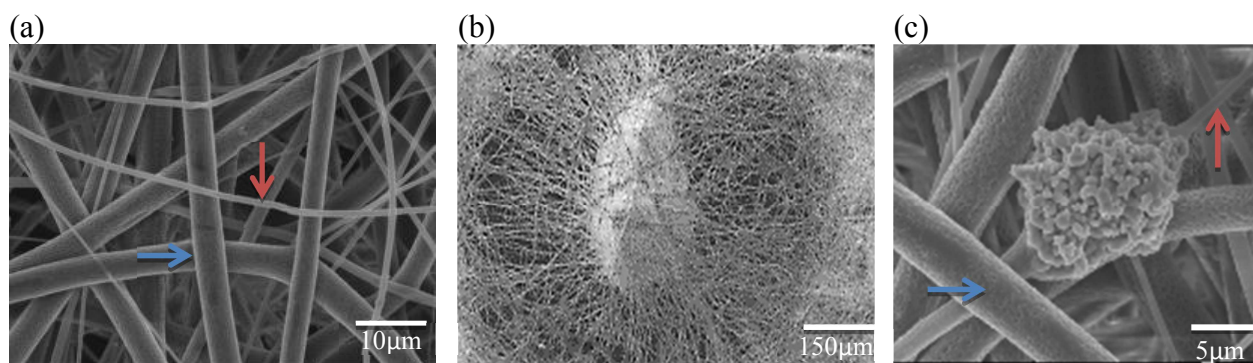
(a)
(b)
(c)

*Figure 2: (a) sample A; (b) sample B Hollow Shells; (c) sample C with BCP micro particles.
Arrows : class I (blue) and class II (red)*

BCP Micro particles of membranes type C were localized by EDX cartography and appeared to be partially encapsulated into fibers leaving the grains and micropores appear (figure 2c). It was pointed out that only fibers of class II contained BCP micro particles (figure 2c). Qualitative EDX spectra of BCP particles confirmed the presence of calcium and phosphate without any contaminants (figure 3).

Figure 3: Qualitative Energy Dispersive X-Ray (EDX) spectra of BCP microparticles (arrow) in scaffold C showed only Ca and P
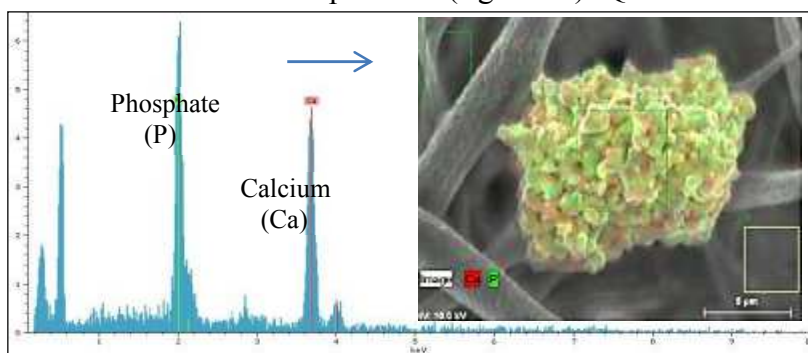
For all types of scaffolds, only class I fibers were characterized as microporous, and image analysis was performed to evaluate surface porosity of these fibers (figure 2 and figure 4). Surface intraporosity of class I fibers were calculated as between 10 and 15 %. Class II thinner fibers presented almost none porosity. The density of all samples was 0.3 g/cm^3 ± 0.05, interporosity between the fibers was calculated from density and rounded to 75 % for all scaffolds types.
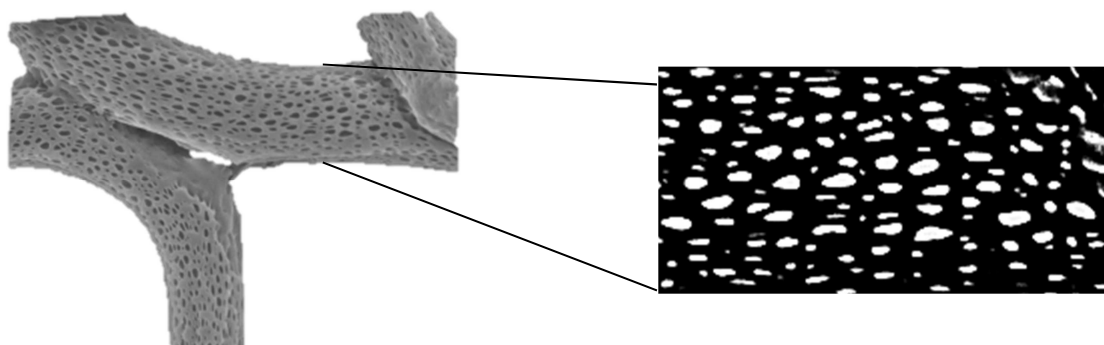

Figure 4: Image analysis of class I fiber surface intraporosity

Confocal microscopy observations of living cells adhering to scaffolds shown two main differences between samples A, C and sample B. The first one was the cell density, which appeared higher inside hollow shells than on fibers (figure 5c). The second one was the cell morphology, filiforms cells were extended along the fibers (figure 5a and 5b) while much more spreaded inside hollow shells as shown in figure 5c.
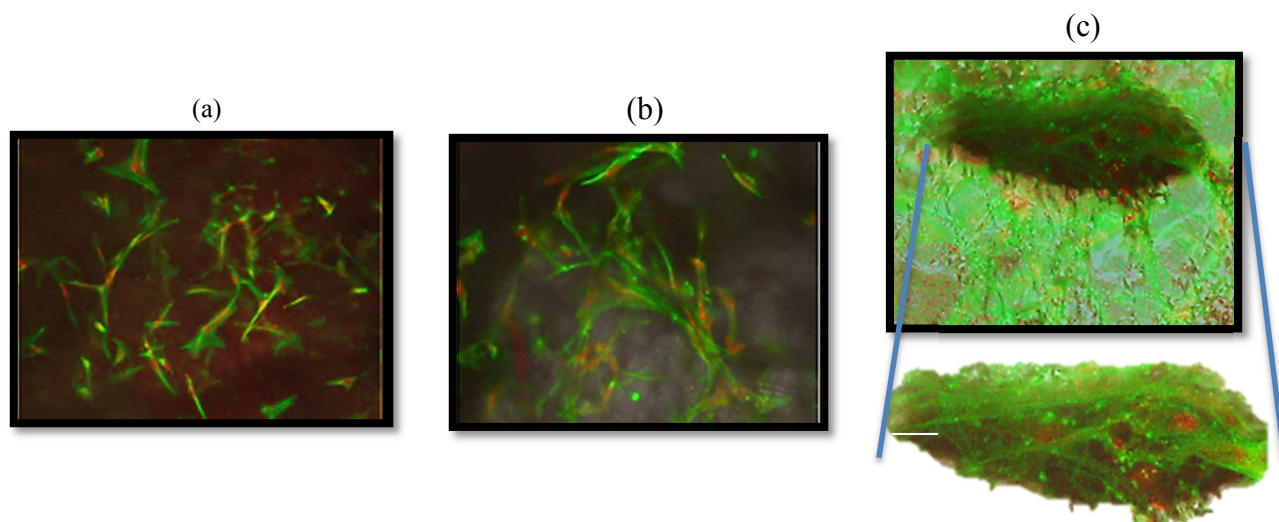
Figure 5: Filiform cells adhering on fibers of samplee A (a), sample B (b), and spreading in hollow shells of sample C (c) at day 3

Discussion

Confocal microscopy observations enabled us to conclude PLDLLA fiber scaffolds are favorable to cell adhesion and proliferation with cell guidance along fibers leading to filiform morphology [26].

The incorporation of BCP micro particles in the polymeric solution lead to alter the homogeneity of the latter and thus disrupt the stability of the Taylor cone at the time of passage of particles through the needle of the syringe. This results in a decrease of the fiber diameter containing the ceramic micro particles. The slight increase in which that did not contain it due to higher magnitude of fluctuation of Taylor cone [27]. This fluctuation phenomenon is the cause of non-homogeneity of fibers diameters observed in this study (class I and class II fibers) [28].

The advantages of embedded BCP hollow granules in PLDLLA electrospun scaffolds are related to the seminal role of macropore concavities in adhesion and cell protection [29-31]. This specific properties combined to microporosity known to enhance proteins adhesion and body fluids penetration [32, 33]. The only matter is to provide better homogeneity around non-conductive ceramic hollow shell which lead to lower fiber density areas as shown in this study. Moreover the high micrometric diameters of obtained fibers involved a better flexibility but a lower tensile strength than nanofibers [34].

Conclusion

Electrospinning has been demonstrated to be a low-cost, fast and easy one-step process to perform promising 3D composite fiber-based scaffolds with cell adhesion and protection enhancement thanks to new Hollow Shells incorporation.All membranes were flexible and easy to handle which are assets to provide relevant scaffolds for clinical purposes particularly in regenerative medicine.

Acknowledgments:

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