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Electrospun poly(ε-caprolactone)/Ca-deficient hydroxyapatite nanohybrids: Microstructure, mechanical properties and cell response by murine embryonic stem cells

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ABSTRACT

Nanohybrid scaffolds mimicking extracellular matrix are promising experimental models to study stem cell behaviour, in terms of adhesion and proliferation. In the present study, the structural characterization of a novel electrospun nanohybrid and the analysis of cell response by a highly sensitive cell type, embryonic stem (ES) cells, are investigated. Ca-deficient hydroxyapatite nanocrystals (d-HAp) were synthesized by precipitation. Fibrous PCL/d-HAp nanohybrids were obtained by electrospinning, d-HAp content ranging between 2 and 55 wt.%. Electrospun mats showed a non-woven architecture, average fiber size was 1.5 ± 0.5 μm, porosity 80–90%, and specific surface area 16 $m^2 g^{-1}$. Up to 6.4 wt.% d-HAp content, the nanohybrids displayed comparable microstructural, mechanical and dynamo-mechanical properties. Murine ES cell response to neat PCL and to nanohybrid PCL/d-HAp (6.4 wt.%) mats was evaluated by analyzing morphological, metabolic and functional markers. Cells growing on either scaffold proliferated and maintained pluripotency markers at essentially the same rate as cells growing on standard tissue culture plates with no detectable signs of cytotoxicity, despite a lower cell adhesion at the beginning of culture. These results indicate that electrospun PCL scaffolds may provide adequate supports for murine ES cell proliferation in a pluripotent state, and that the presence of d-HAp within the mat does not interfere with their growth.

1. Introduction

The composition and topology of the extracellular matrix (ECM) has been found to affect cell morphology, function, and physiological response [1]. Electrospun nanofibrous scaffolds aimed to mimic the architecture and biological functions of ECM, are considered as very promising substrates for tissue engineering [2,3]. Among them, scaffolds made of poly(ε-caprolactone) (PCL), a bioresorbable aliphatic polyester, have been used to provide a 3D environment for *in vitro* embryonic stem cell culture and differentiation [4]. The use of a 3D environment might make the culture system closer to the *in vivo* situation: how various 3D scaffolds might affect the differentiation potential of murine ES cells has been the object of recent research [5–7]. Electrospun hybrid scaffolds based on bioresorbable polymers and conventional hydroxyapatite allow osteoblast proliferation and differentiation, and are thus considered very promising as bone scaffolding materials [8–10]. It is well known that Ca-deficient-hydroxyapatite (d-HAp) shows more similar features to biological apatites with respect to conventional stoichiometric hydroxyapatite (s-Hap) [11]. In the present study we have evaluated the biocompatibility of PCL/d-HAp nanohybrid mats by using murine ES cells. Because of their high sensitivity to toxic agents [12], their embryonic origin [13,14] and their ability to differentiate along all tissue types [15,16], ES cells are already being used in a standard cytotoxicity and embryotoxicity test for soluble compounds [17,18], and might as well provide a good model to test biocompatibility *in vitro* of novel scaffolds. In greater detail, we have analyzed how fibrous PCL/HAp affects an essential property of ES cells: their ability to proliferate without differentiating. Fibrous PCL/d-HAp mats were obtained by electrospinning, with a d-HAp content ranging from 2 to 55 wt.%. The d-HAp nanopowder was freshly synthesized by precipitation. Microstructure, mechanical and dynamo-mechanical properties of the electrospun nanohybrid mats were extensively investigated. The biocompatibility of the mats was evaluated by culturing mouse ES cells and analyzing colony morphology and number, ES cell adhesion, viability, proliferation and preservation of pluripotency markers. To our knowledge, this is the first report in which electrospun PCL

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nanohybrids containing d-HAp have been described, and their biocompatibility tested by the use of ES cells.

2. Experimental

2.1. Synthesis of Ca-deficient hydroxyapatite (d-HAp) nanopowders

The d-HAp nanopowder was prepared in a double-walled jacket reactor at 40 °C following a precipitation route described elsewhere [19]. Stoichiometric volume of 1M calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$, 99.2% *Aldrich*) was added dropwise to diammonium hydrogen phosphate ($(NH_4)_2HPO_4$, 99.2% *Aldrich*) pH was continuously monitored and adjusted at 9.0 ± 0.1 by adding NH_4OH conc. Precipitate was aged in mother solutions for 24 h, washed with NH_4OH aqueous solution, and vacuum filtered. Chemical analysis was performed by induced coupled plasma atomic emission spectroscopy (*AES-ICP, JobinYvon JV 24R*). Morphology was examined by transmission electron microscopy in bright field mode, accelerating voltage being 100 kV (*TEM, Philips CM120*) and specific surface area measured by N_2 adsorption (*Sorptomatic 1900, Carlo Erba*) using the Brunauer–Emmett–Teller (BET) method. X-ray diffraction analysis (XRD) (*Philips X'Pert*) was performed in the following conditions: Cu K_α radiation $\lambda = 1.5405600$ Å, 20–55° 2θ, step size 0.010°, time per step 2 s, scan speed 0.005°/s.

2.2. Fabrication of electrospun PCL membranes

PCL (*Aldrich*, MW 80000) was dissolved at RT under magnetic stirring in a 1:1 dichloromethane (CH_2Cl_2) and N,N-dimethylformamide (DMF) mixture, the polymer concentration being 12% (w/v). The solution was poured in a glass syringe (*Socorex*) equipped with a 21 G needle, fixed in a digitally controlled syringe pump (*KD Scientific*) and electrospun as follows: tension 21 kV (*Spellman, Model SL 30*), needle-target distance 10 cm, feed rate 1 ml h^{-1}. According to previous studies [20–23], electrospun meshes were dried under vacuum for 24 h.

2.3. Fabrication of electrospun PCL/d-HAp membranes

The d-HAp nanopowder was poured to a 1:1 CH_2Cl_2–DMF solution and the suspension ultrasonicated for 2 h. PCL granules were then added and the mixture stirred for 36 h. Suspensions were electrospun and the resulting mats treated as described above. All prepared PCL/d-HAp nanohybrid samples are listed in Table 1.

2.4. Viscosity and conductivity

Viscosity of polymeric suspensions was measured at 25 °C by digital viscosimeter (*Brookfield DV-II+*) equipped with a SC4-21 spindle at 20 rpm and electrical conductivity measured at 25 °C by conductivity meter (*CDM230, Analitica De Mori*).

Table 1
Designation and composition of PCL/d-HAp nanohybrid electrospun mats.

Sample	PCL/d-Hap, wt.%[a]	PCL/d-Hap, vol.%[b]
PCL	–	–
2HAp	2.0	5
4HAp	3.6	9
6HAp	6.4	16
25HAp	24.9	48
40HAp	42.5	67
55HAp	55.1	77

[a] Values referred to residual mass ($T = 600$ °C), as calculated by TGA measurements.
[b] Volume fraction of the ceramic filler (ϕ_c) was derived from the weight percentage (W_c) as follows, where ρ_p e ρ_c are, respectively the density of PCL (ρ_{PCL} 1.145 g cm^{-3}) and d-HAp ($\rho_{d\text{-}HAp}$ 3.156 g cm^{-3}): $\frac{1}{\phi_c} = 1 + \frac{\rho_p}{\rho_c}\left(\frac{100}{P_c} - 1\right)$.

2.5. Scanning electron microscopy (SEM) and energy dispersion spectroscopy (EDS)

Microstructure of electrospun fabrics was investigated by SEM (*Cambridge Stereoscan 300*). The actual d-HAp distribution was evidenced by energy dispersion spectroscopy (EDS). Average fiber diameter was determined on 120 different fibers by means of CAD software.

2.6. Density, porosity and specific surface area

Density (ρ) was estimated as mass to volume ratio on 12 mm diameter disks cut out of the membranes, four samples were considered for each material. Sample thickness was measured according to ISO7198 by means of a digital micrometer equipped with a 2 kg load cell. Porosity (ε) was estimated as follows, where ρ_0 is the density of as-purchased PCL (1.145 g cm^{-3}):

$$\varepsilon = \left(1 - \frac{\rho}{\rho_0}\right) \cdot 100 \quad (1)$$

Specific surface area was measured by N_2 adsorption (*Sorptomatic 1900, Carlo Erba*) using BET method.

2.7. Infrared spectroscopy (ATR-IR) and thermal analyses

IR spectra were recorded in the 4000–400 cm^{-1} region by means of a *Jasco FTIR-615* spectrophotometer in ATR reflection method, spectral resolution 4 cm^{-1}.

Thermogravimetric analysis (TGA) was performed by means of a quartz rod microbalance (*Seiko Exstar 6000*). Measurements were performed in N_2 atmosphere between 30 and 1000 °C, heating rate 10 °C min^{-1}. Residual mass and maximum degradation temperature (T_d) were measured. Thermal characterization was carried out using Differential Scanning Calorimeter (DSC) (*Perkin Elmer Pyris 1*), heating and cooling scans were performed in the following conditions: sample weight 10 mg, heating rate 10 °C min^{-1}, temperature range from −25 °C to 100 °C, N_2 atmosphere. Melting temperature (T_m), melting enthalpy (ΔH_m), and crystallinity degree (X_c) were determined from the heating scan. Crystallization temperature (T_c) and enthalpy (ΔH_c) were measured from the cooling run. X_c of the PCL component in the nanocomposites was calculated according to (2):

$$X_c(\%) = \Delta H_m / \Delta H_0 \quad (2)$$

where ΔH_m is derived from the DSC curves, with respect to the actual PCL content [24], ΔH_0 is the theoretical enthalpy of the fully crystalline polymer, for PCL the considered value was 136 J g^{-1} [25].

2.8. Mechanical characterization

Uniaxial tensile tests were carried out on dog-bone specimens, gauge width 4.8 mm and gauge length 22.3 mm. Mechanical tests were performed at 1.2 mm min^{-1} to rupture by an electromechanical machine equipped with a 5 kg load cell (*TA-HDi, Stable Micro Systems*). Four specimens were considered for each electrospun matrix. The tensile modulus (E) was evaluated between 0 and 5% strain [26]. The yield stress was determined from the intersection of the stress–strain curve with a line parallel to the linear region and offset by 2% strain. The ultimate stress (σ_{max}) and ultimate percent deformation (ε_f) were calculated considering the nominal cross-sectional area of the tensile specimen. Dynamic-Mechanical Analysis (DMA) was performed using a *Reometric Scientific-ARES* sample size was 10 mm width, 30 mm length, 0.3 mm thick. Shear modulus was